Characterization of recombinant human glucuronyltransferase I involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans

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Abstract We characterized the recombinant glucuronyltransferase I (GlcAT-I) involved in the glycosaminoglycan-protein linkage region biosynthesis. The enzyme showed strict specificity for Galβ1-3Galβ1-4Xyl, exhibiting negligible incorporation into other galactoside substrates including Galβ1-3Galβ1-*O*-benzyl, Galβ1-4GlcNAc and Galβ1-4Glc. A comparison of the GlcAT-I with another β1,3-glucuronyltransferase involved in the HNK-1 epitope biosynthesis revealed that the two β1,3-glucuronyltransferases exhibited distinct and no overlapping acceptor substrate specificities in vitro. Nevertheless, the transfection of the GlcAT-I cDNA into COS-1 cells induced the significant expression of the HNK-1 epitope. These results suggested that the high expression of the GlcAT-I gene rendered the cells capable of synthesizing the HNK-1 epitope.

Key words: Glucuronyltransferase; Glycosaminoglycan; HNK-1 epitope; Proteoglycan; Recombinant enzyme

1. Introduction

Proteoglycans are polyanionic molecules that are distributed on the surfaces of most cells and the extracellular matrices in virtually every tissue, and comprise a wide range of structures involving different core proteins and different classes, numbers, and lengths of sulfated glycosaminoglycans (GAGs) (for reviews, see [1,2]). Despite the ubiquity of this family of molecules, a wide variety of proteoglycans with characteristic sulfated GAG chains exhibit tissue-specific and developmentally regulated expression (for a review, see [3]), and have been implicated in the regulation and maintenance of cell proliferation, cytodifferentiation, and tissue morphogenesis (for a review, see [4]).

Sulfated GAGs including heparin/heparan sulfate, chondroitin sulfate and dermatan sulfate are covalently bound to Ser residues in the core proteins through the common carbohydrate-protein linkage structure, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser [1,2]. The linkage region synthesis is initiated by the addition of Xyl to specific Ser residues in the core protein followed by the addition of two Gal residues, and is completed by the addition of a GlcA residue. GAGs are built up on this linkage region by the alternating addition of *N*-acetylhexosamine and GlcA residues. Heparin/heparan sulfate is synthesized once GlcNAc is transferred to the common linkage region, while chondroitin sulfate is formed if GalNAc is first added. The addition of each sugar is thought to be catalyzed by a specific glycosyltransferase, which uses the corresponding UDP-sugar as a donor substrate [1,2].

The glucuronyl transfer to the Gal residue, the final biosynthetic step of the common linkage region, is catalyzed by a key enzyme, β1,3-glucuronyltransferase, that has been termed glucuronyltransferase I (GlcAT-I). We recently cloned the cDNA encoding the GlcAT-I from human placenta [5] based upon the information of the amino acid sequence alignment of rat glucuronyltransferase (GlcAT-P) involved in the biosynthesis of the carbohydrate epitope HNK-1 with putative proteins in *Caenorhabditis elegans* and *Schistosoma mansoni* [6]. The properties of GlcAT-I have not yet been described in detail since attempts to purify GlcAT-I to homogeneity have not been successful due to the low concentrations and the difficulty in solubilizing the enzyme. In the present study, the properties and substrate specificities of the recombinant GlcAT-I were determined and compared with those of the recombinant GlcAT-P.

2. Materials and methods

2.1. Materials

UDP-[*U*-^{14}C]GlcA (285.2 mCi/mmol) and unlabeled UDP-GlcA were purchased from New England Nuclear (Boston, MA, USA) and Sigma (St. Louis, MO, USA), respectively. Recombinant *N*-glycanase (*N*-glycosidase F; EC 3.5.1.52) was obtained from Boehringer Mannheim (Mannheim, Germany). Chondroitin (a chemically desulfated derivative of whale cartilage chondroitin sulfate A), Galβ1-4Glc, Galβ1-4GlcNAc, and the monoclonal antibody (HepSS-1) were purchased from Seikagaku Corp. (Tokyo, Japan). Galβ1-3GlcNAc and Galβ1-3GalNAc were obtained from Sigma. Galβ1-3Galβ1-4Xyl was a kind gift from Dr. N.B. Schwartz (University of Chicago, Chicago, IL, USA). Galβ1-3Galβ1-*O*-benzyl and Galβ1-4GlcNAcβ1-*O*-naphthalenemethanol [7] were kindly provided by Dr. J.D. Esko (University of California, San Diego, CA, USA).

2.2. Expression of the soluble forms of GlcAT-I and GlcAT-P and enzyme assay

The construction of soluble forms of GlcAT-I and GlcAT-P fused with the cleavable insulin signal sequence and the protein A IgG-binding domain was carried out as described, respectively [5,6]. Each expression plasmid (10 μg) was transfected into COS-1 cells on 100-mm plates using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD, USA) according to the instructions provided by the manufacturer. Two days after transfection, 1 ml of the culture

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Abbreviations: FITC, fluorescein isothiocyanate; GlcA, D-glucuronic acid; GlcAT-I, glucuronyltransferase I (EC 2.4.1.135); GlcAT-P, glycoprotein-specific glucuronyltransferase; GAG, glycosaminoglycan; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction

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medium was collected and incubated with 10 μl of IgG-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4°C. The beads recovered by centrifugation were washed with and then resuspended in each assay buffer, and tested for glucuronyltransferase activities using oligosaccharides (1 nmol each), chondroitin (300 μg), and asialoorosomucoid (20 μg) as acceptor substrates as described, respectively [8–10]. As shown in Section 3, the standard assay conditions for GlcAT-I were established by examining various factors such as buffers, metal ions and substrate concentrations using Galβ1-3Galβ1-4Xyl as an acceptor. The standard assay mixture contained 10 μl of the resuspended beads, 1 nmol of Galβ1-3Galβ1-4Xyl, 14.3 μM UDP-[^{14}C]GlcA (1.66×10^5 dpm), 50 mM MES buffer, pH 6.5 and 2 mM $MnCl_2$ in a total volume of 30 μl. Reaction mixtures were incubated at 37°C for 4 h, and then ^{14}C-labeled products were separated from UDP-[^{14}C]GlcA by passage of the reaction mixture diluted in 1 ml 5 mM sodium phosphate, pH 6.8, through Pasteur pipette columns containing Dowex 1-X8 (a PO_4^{2-} form, 100–400 mesh: Bio-Rad, Tokyo, Japan). The isolated products were quantified by scintillation spectrophotometry. The net [^{14}C]GlcA incorporation into Galβ1-3Galβ1-4Xyl was calculated by subtraction of the blank value obtained in its absence. Under the established incubation conditions for GlcAT-I, GlcA incorporation into Galβ1-3Galβ1-4Xyl was proportional to the incubation period for up to 4 h (data not shown).

2.3. Immunoblotting

The purified GlcAT-I was digested with *N*-glycanase according to the instructions provided by the manufacturer. After digestion, the reaction mixture and the undigested sample were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20 and 1% bovine serum albumin and then incubated with the diluted antiserum (1:200) raised in rabbits against the motif IV peptide EPRAANCTRVLVWHTRTEKP of the human GlcAT-I [5]. Bound antibodies were visualized with an enzyme reaction using alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, Madison, WI, USA) as a secondary antibody.

2.4. Construction of expression vector containing a full-length GlcAT-I cDNA

The full-length GlcAT-I was amplified by polymerase chain reaction (PCR) using a 5′-specific primer (5′-GCTCTAGAGCGGCCATGAAGCTGAA-3′) containing an *Xba*I site and the start codon and a 3′-specific primer (5′-GCTCTAGAGCCTGAAAAGAGGTGGTAG-3′) containing an *Xba*I site located 40 bp downstream of the stop codon. PCR reactions were carried out with *Pfu* polymerase (Stratagene, La Jolla, CA, USA) with 30 cycles of a reaction consisting of 30 s of denaturation at 96°C, 45 s of annealing at 60°C, and 90 s of elongation at 72°C. The PCR fragment was subcloned into the *Xba*I site of pEF-BOS [6].

2.5. Expression of the full-length GlcAT-I and GlcAT-P cDNAs in COS-1 cells

COS-1 cells were seeded on tissue culture dishes (100 mm) and transfected with 10 μg of the full-length GlcAT-I cDNA in pEF-BOS or the full-length GlcAT-P cDNA in pEF-BOS [6] using LipofectAMINE (Life Technologies) according to the instructions provided by the manufacturer. After 48 h, the COS-1 cells were washed with phosphate-buffered saline, scraped off the dishes and homogenized in 1.0 ml/dish of 50 mM MES buffer, pH 6.5 containing 1.0% Triton X-100 and Complete (EDTA-free protease inhibitors; Boehringer Mannheim). The homogenates were used as the enzyme source. For fluorescent immunostaining, the COS-1 cells were fixed with methanol for 20 min at −20°C, blocked with 3% bovine serum albumin for 30 min, incubated with 10 μg/ml of the HNK-1 antibody [6] or of HepSS-1 for 2 h at room temperature and then visualized with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM. These cells were observed using a confocal laser scan microscope, Fluoview (Olympus, Tokyo, Japan).

3. Results

3.1. Expression of a soluble form of the GlcAT-I

To facilitate the functional analysis of the glucuronyltransferase, a soluble form of the protein was generated by replacing the first 43 amino acids of the glucuronyltransferase with a cleavable insulin signal sequence and a protein A IgG-binding domain as described [5], since the enzyme is tightly bound to membranes [8]. When the expression plasmid containing the GlcAT-I/protein A fusion was expressed in COS-1 cells, approximately 75-kDa and 65-kDa proteins were secreted (Fig. 1, lane 1). The apparent M_r of the 75-kDa fused protein was reduced to 65 kDa after *N*-glycanase treatment (Fig. 1, lane 2), indicating that the one potential *N*-linked glycosylation site of the GlcAT-I is partly being utilized. The fused enzyme expressed in the medium was absorbed on IgG-Sepharose beads to eliminate endogenous glucuronyltransferase, and then the enzyme-bound beads were used as an enzyme source for further studies.

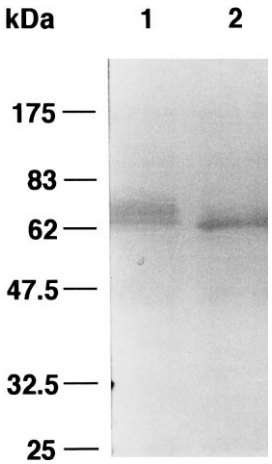

Fig. 1. Expression of a soluble protein A/GlcAT-I fusion protein and *N*-glycanase digestion of the fusion protein. Western blot of the purified recombinant GlcAT-I (lane 1) and the *N*-glycanase digest of the recombinant GlcAT-I (lane 2) separated by 10% SDS-PAGE was incubated with the antiserum raised against the motif IV peptide of the human GlcAT-I as described in Section 2.

3.2. Properties of the recombinant GlcAT-I activity

Fig. 2 shows the effects of buffers and pH on the recombinant GlcAT-I. The activity was maximal at pH 6.5. The enzyme activity was somewhat affected by the buffers used. MES buffer allowed over 1.4-fold more activity than HEPES buffer at pH 6.5 (Fig. 2). Divalent cations were essential for the enzymatic reaction, and 10 mM EDTA completely abolished the activity (Fig. 3A). Mn^{2+} exhibited the highest activity under the standard assay conditions, and Mg^{2+} and Cu^{2+} were only 5.6 and 2.7% as effective as Mn^{2+}, respectively (Fig. 3A). The optimal concentration of Mn^{2+} was about 2 mM (Fig. 3B).

To investigate the effects of concentrations of the acceptor Galβ1-3Galβ1-4Xyl and the donor UDP-GlcA, some of the kinetic parameters of the GlcAT-I were determined. The apparent K_m values for Galβ1-3Galβ1-4Xyl and UDP-GlcA were 80.4 μM and 29.3 μM, respectively (data not shown).

3.3. In vitro acceptor specificities of the GlcAT-I and GlcAT-P

To distinguish the specificity of the GlcAT-I from that of another β1,3-glucuronyltransferase, GlcAT-P, which has been cloned, recombinant GlcAT-I was compared with the recombinant form of GlcAT-P for its utilization of a variety

of acceptor substrates containing a terminal Gal or GalNAc residue. An initial examination of GlcAT-I with oligosaccharide substrates revealed that Galβ1-3Galβ1-4Xyl was the best substrate and the apparent K_m value for the acceptor substrate was 80.4 μM as described above. Accordingly, we decided to compare the specificities of the two enzymes at a fixed concentration of 33 μM for various acceptor substrates. As shown in Table 1, the GlcAT-I utilized only Galβ1-3Galβ1-4Xyl, and little incorporation was observed with other substrates containing a terminal GalNAc or Gal residue, i.e. polymer chondroitin, *N*-acetyllactosamine, lactose, asialoorosomucoid, Galβ1-3GlcNAc, Galβ1-3GalNAc and notably Galβ1-3Galβ1-*O*-benzyl. These findings indicated that the minimum structural requirement for the acceptor substrate of the GlcAT-I was the trisaccharide sequence Galβ1-3Galβ1-4Xyl, and the enzyme recognized up to the third saccharide residue (Xyl) from the non-reducing end. In contrast, the GlcAT-P preferentially utilized substrates containing the terminal Galβ1-4GlcNAc structure, i.e. Galβ1-4GlcNAcβ1-*O*-naphthalenemethanol, *N*-acetyllactosamine and asialoorosomucoid, whereas little incorporation was observed with other substrates, i.e. chondroitin, Galβ1-3Galβ1-4Xyl, Galβ1-3GlcNAc, Galβ1-3GalNAc and remarkably, lactose. These findings indicated that the minimum structural requirement for the acceptor substrate of the GlcAT-P was the disaccharide sequence Galβ1-4GlcNAc, and the enzyme recognized the *N*-acetyl group of the GlcNAc residue. These results together indicate that the substrate specificity of the GlcAT-I is clearly distinct from that of the GlcAT-P in vitro.

3.4. *Transfection of the full-length GlcAT-I cDNA into COS-1 cells*

In view of the fact that the transfection of the full-length GlcAT-P cDNA into COS-1 cells induced the expression of the HNK-1 epitope [6], it was of interest to determine whether the GlcAT-I cDNA transfectant induces the expression of the HNK-1 epitope. Thus, the full-length GlcAT-I cDNA was transfected into COS-1 cells to test the substrate specificity

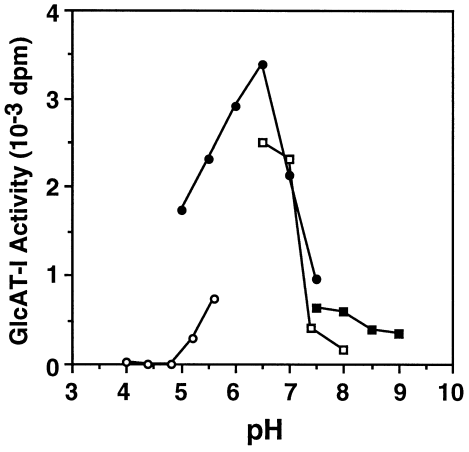

Fig. 2. Effects of buffers and pH on the activity of GlcAT-I. The effects of pH on the GlcA transfer to Galβ1-3Galβ1-4Xyl were determined under standard assay conditions with different buffers at a final concentration of 50 mM. The buffers were sodium acetate (○), MES-NaOH (●), HEPES-NaOH (□), and Tris-HCl (■). Assays proceeded as described in Section 2. Data represent one of two series of independent experiments, where the two series of experiments gave essentially identical results.

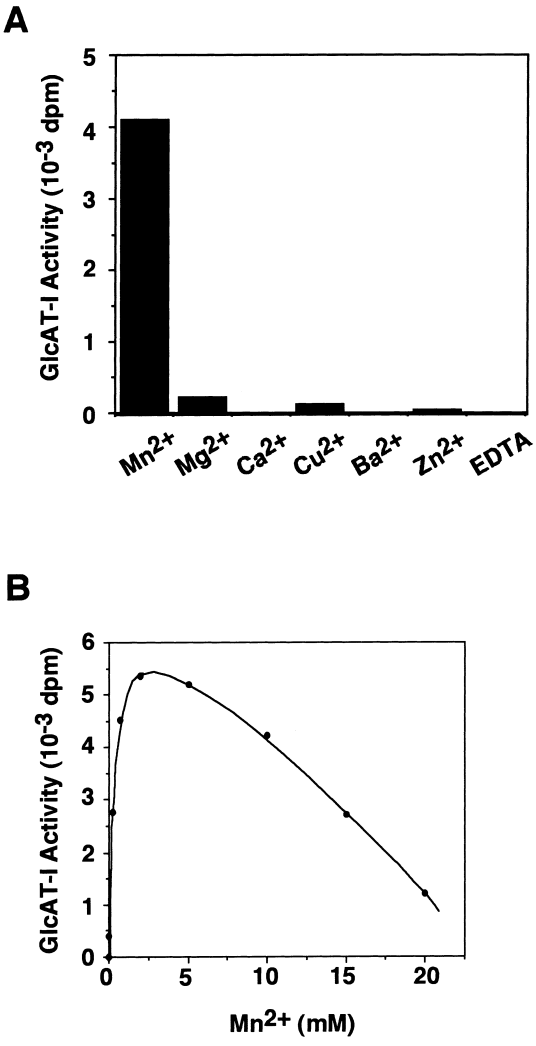

Fig. 3. Effects of divalent cations and Mn^{2+} concentration on the GlcAT-I activity. A: The effects of divalent cations on the GlcA transfer to Galβ1-3Galβ1-4Xyl were determined under standard assay conditions with different divalent cations or EDTA at a final concentration of 10 mM. B: The effects of Mn^{2+} concentrations on the GlcA transfer to Galβ1-3Galβ1-4Xyl were determined under standard assay conditions, except that the concentration of $MnCl_2$ was varied. Assays proceeded as described in Section 2. Data represent one of two series of independent experiments, where the two series of experiments gave essentially identical results.

of the intracellular GlcAT-I. As a control, the expression of heparan sulfate was examined using the anti-heparan sulfate monoclonal antibody HepSS-1. The expression levels of heparan sulfate of both GlcAT-I and GlcAT-P transfectant were comparable to that of the mock-transfected cells (Fig. 4A–C). Unexpectedly, the transfection of the GlcAT-I cDNA into COS-1 cells induced a significant expression of the HNK-1 epitope, although the intensity was somewhat weaker than that of the GlcAT-P transfectant, as demonstrated by fluorescent immunostaining using an HNK-1 antibody followed by an FITC-conjugated secondary antibody (Fig. 4E and F). In contrast, the mock-transfected cells were not stained at all under the conditions used (Fig. 4D), although the cells indeed exhibited the endogenous GlcAT-I gene expression and GlcAT-I activity (Table 2). To exclude the possibility that the full-length membrane-bound forms of GlcAT-I and

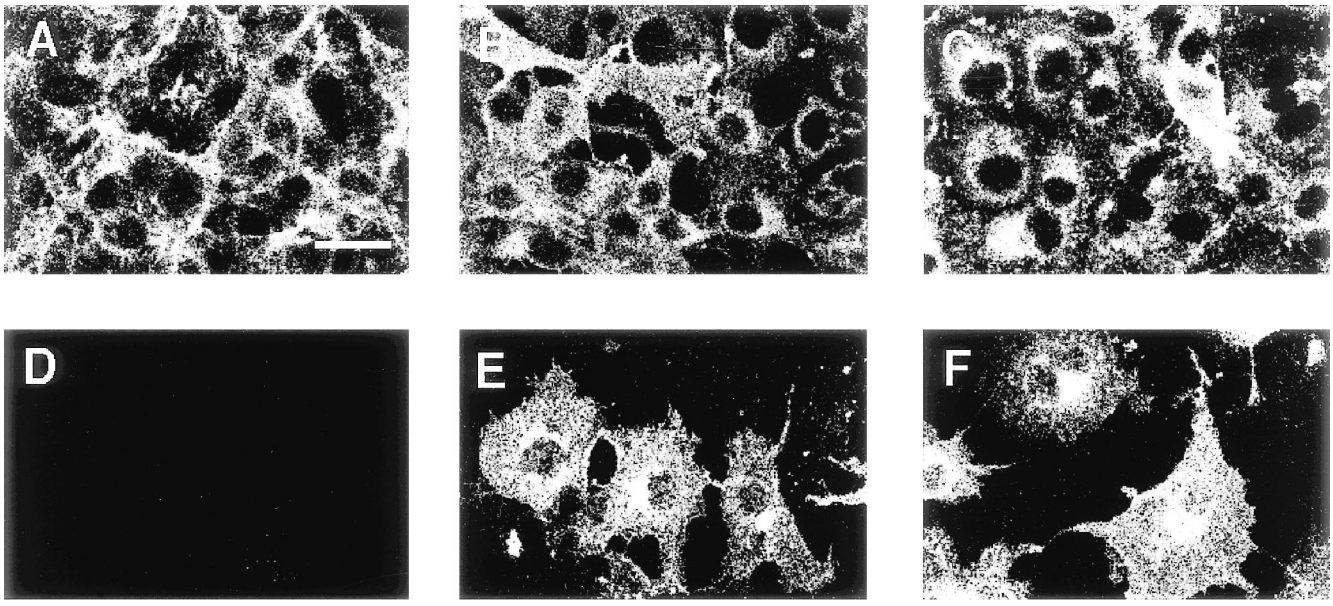

Fig. 4. Immunofluorescence staining of transfected COS-1 cells by HepSS-1 and HNK-1 antibodies. COS-1 cells were transfected with the pEF-BOS (A and D), the GlcAT-I cDNA in pEF-BOS (B and E) and the GlcAT-P cDNA in pEF-BOS (C and F), respectively, as described in Section 2. Indirect immunofluorescence staining of HepSS-1 (A–C) and HNK-1 epitopes (D–F). Scale bar, 200 μm.

GlcAT-P show different substrate specificities from those of their soluble forms that lack both the cytoplasmic and the transmembrane domains, the extracts of the full-length GlcAT-I and GlcAT-P cDNA-transfected cells were assayed for glucuronyltransferase activity using various acceptor substrates. As shown in Table 2, the GlcAT-I and GlcAT-P transfectants surely overexpressed the GlcAT-I and GlcAT-P activities, respectively, and the substrate specificities of the expressed activities in these two full-length transfectants were virtually identical to those of the corresponding soluble forms (see Table 1). Therefore, these results altogether suggested that the high expression of the GlcAT-I gene seemed to render the cells capable of synthesizing the HNK-1 epitope by an as yet undefined mechanism.

4. Discussion

GlcAT-I activity was first detected in an embryonic chick cartilage extract [8] and was subsequently partially purified from embryonic chick brain [11] and mouse mastocytoma cells [12]. However, attempts to purify GlcAT-I to homogeneity have not been successful due to the low concentrations and the difficulty in solubilizing the enzyme. In this study, we investigated the properties of recombinant GlcAT-I, which was expressed in COS-1 cells as a soluble protein A chimeric form and purified using IgG-Sepharose, and the substrate specificity of the purified GlcAT-I was compared with that of recombinant purified GlcAT-P. Our characterization of the purified recombinant GlcAT-I revealed an Mn^{2+} requirement for maximal activity and a pH optimum at pH 6.5, basically in accordance with the results obtained using a particulate fraction prepared from embryonic chick cartilage as an enzyme source [8]. However, in contrast to the previous findings that the reaction occurred over a wide pH range with a poorly defined maximum at pH 5.4 and reached a saturation level at 15 mM Mn^{2+} [8], the recombinant GlcAT-I exhibited a relatively sharp maximum at pH 6.5, although the enzyme activity was somewhat affected by the buffers used (Fig. 2) and the reaction was stimulated by Mn^{2+} up to 2 mM and decreased at higher levels (Fig. 3B). In addition, the K_m value of the recombinant enzyme for UDP-GlcA was low (29.3 μM) compared with the value (100 μM) of the chick cartilage en-

Table 1
Comparison of the acceptor specificity of GlcAT-I and GlcAT-P secreted into the culture medium by transfected COS-1 cells

Acceptor	Enzyme activity[b]	
	GlcAT-I (pmol/ml medium/h)	GlcAT-P (pmol/ml medium/h)
Galβ1-3Galβ1-4Xyl	14.2	0.5
Galβ1-3Galβ1-*O*-benzyl	n.d.[c]	n.d.
Chondroitin (GalNAcβ1-4GlcA)$_n$	n.d.	1.2
Lactose (Galβ1-4Glc)	n.d.	n.d.
N-Acetyllactosamine (Galβ1-4GlcNAc)	n.d.	14.9
Asialoorosomucoid (Galβ1-4GlcNAc-R)[a]	n.d.	71.6
Galβ1-4GlcNAcβ1-*O*-naphthalenemethanol	n.d.	83.3
Galβ1-3GlcNAc	n.d.	n.d.
Galβ1-3GalNAc	0.2	n.d.

[a]R represents the remainder of the *N*-linked oligosaccharide chain.
[b]The values represent the averages of two independent experiments.
[c]n.d., not detected (<0.1 pmol/ml medium/h).

Table 2
Comparison of the acceptor specificity of GlcAT-I and GlcAT-P in transfected COS-1 cells

Acceptor	Enzyme activity[b]		
	Mock (pmol/mg protein/h)	GlcAT-I (pmol/mg protein/h)	GlcAT-P (pmol/mg protein/h)
Galβ1-3Galβ1-4Xyl	57.7	743.7	53.7
N-Acetyllactosamine (Galβ1-4GlcNAc)	n.d.[c]	n.d.	76.4
Asialoorosomucoid (Galβ1-4GlcNAc-R)[a]	n.d.	n.d.	765.9
Galβ1-3GlcNAc	n.d.	n.d.	n.d.
Galβ1-3GalNAc	n.d.	2.5	n.d.

[a]R represents the remainder of the *N*-linked oligosaccharide chain.
[b]The values represent the averages of two independent experiments.
[c]n.d., not detected (<0.5 pmol/mg protein/h).

zyme, although Galβ1-3Gal was used as an acceptor in that study [8] instead of Galβ1-3Galβ1-4Xyl.

Our previous characterization of the acceptor substrate specificity of recombinant GlcAT-I revealed that the enzyme showed a strict specificity toward the linkage trisaccharide-serine, Galβ1-3Galβ1-4Xylβ1-*O*-Ser [5]. Neither longer linkage oligosaccharide-serines nor polymer chondroitin was utilized as an acceptor substrate. These findings clearly indicate that the transfer of GlcA to the linkage trisaccharide primer is mediated by GlcAT-I, distinct from the enzyme that has been termed glucuronyltransferase II involved in the formation of the repeating disaccharide units of chondroitin sulfate, as proposed previously [8,13]. Helting and Rodén [8] reported that linkage region oligosaccharides such as Galβ1-3Gal and Galβ1-3Galβ1-4Xyl as well as Galβ1-3Galβ1-4Xylβ1-*O*-Ser were good acceptors for GlcAT-I in a particulate enzyme system prepared from embryonic chick cartilage. In fact, the recombinant GlcAT-I efficiently utilized Galβ1-3Galβ1-4Xyl as an acceptor (the K_m value was 80.4 μM), consistent with the property of the previously characterized enzyme and with the finding that the serine residue is not essential for the GlcA transfer. However, in contrast to the previous findings that the disaccharide Galβ1-3Gal was a good acceptor [8,12], the recombinant GlcAT-I did not utilize Galβ1-3Galβ1-*O*-benzyl linked to an artificial aglycon as an acceptor under our assay conditions (Table 1), indicating that the enzyme recognized up to the third saccharide residue (Xyl) from the non-reducing end. The trisaccharide recognition deduced from the present study argues against the recent report by Wei et al. [14] who described that Galβ1-3Galβ1-*O*-naphthalenemethanol and Galβ1-3Galβ1-*O*-benzyl served as acceptors for the recombinant hamster GlcAT-I which they obtained by expression cloning.

The crude enzyme preparations including the embryonic chick cartilage particulate fraction were shown to catalyze the GlcA transfer not only to GAG-protein linkage region fragments containing the characteristic structure (e.g. Galβ1-3Galβ1-4Xyl) but also to disaccharides with analogous structures such as lactose (Galβ1-4Glc) and *N*-acetyllactosamine (Galβ1-4GlcNAc) [8,11,12]. In strong contrast, neither lactose nor *N*-acetyllactosamine was utilized by the recombinant enzyme in the present study (Table 1). Curenton et al. [15] suggested that these glucuronyl transfer reactions might be catalyzed by two distinct β1,3-glucuronyltransferases, GlcAT-I and GlcAT-P. This hypothesis was in part confirmed by the present characterization of these two recombinant enzymes. However, considering the present finding that neither the recombinant GlcAT-I nor GlcAT-P transferred GlcA to lactose, it is likely that a third member of the β1,3-glucuronyltransferase family that utilizes lactose as an acceptor substrate exists.

In this study, we demonstrated that GlcAT-I and GlcAT-P exhibited distinct and no overlapping acceptor substrate specificities in vitro. Nevertheless, the transfection of GlcAT-I cDNA into COS-1 cells induced the expression of the HNK-1 epitope, although the intensity was somewhat weaker than that of the GlcAT-P transfectant (Fig. 4). Although the seeming discrepancy between the in vitro and in vivo substrate specificities remains to be clarified, similar phenomena were observed for other glycosyltransferases including sialyltransferases (for a review, see [16]). When vectors are used under the control of a strong promoter to express an exogenous cDNA, a significant amount of the protein is occasionally expressed in the cells [16]. If a large amount of an enzyme is expressed in the Golgi apparatus, a negligible in vitro activity may become meaningful. On the basis of these results, an incorrect conclusion may be drawn. In the present case, considering that the mock-transfected cells were not stained at all with the HNK-1 antibody although the cells indeed exhibited the endogenous GlcAT-I gene expression and GlcAT-I activity (Table 2), it is reasonable to speculate that the GlcAT-I does not participate in the formation of the HNK-1 epitope under physiological conditions. In fact, the GlcAT-I gene is weakly, but ubiquitously expressed in virtually every human tissue examined (Y. Tone, H. Kitagawa, and K. Sugahara, unpublished results), which is in accordance with the observations that proteoglycans are distributed on the surfaces of most cells and the extracellular matrices in virtually every tissue [1,2], but in contrast to the findings that the expression of the HNK-1 epitope is highly restricted and is temporally and spatially regulated during the development of the nervous system [17]. Therefore, GlcAT-I seems to be mainly involved in the biosynthesis of the GAG-protein linkage region of proteoglycans. However, based on the present findings that the transfection of GlcAT-I cDNA into COS-1 cells induced a significant expression of the HNK-1 epitope, it is possible that tissues which abundantly express the GlcAT-I gene synthesize the HNK-1 epitope GlcA(3-*O*-sulfate)β1-3Galβ1-4GlcNAc, especially since the HNK-1 3-*O*-sulfotransferase is widely distributed in various tissues and GlcAT-P most likely regulates the expression of the HNK-1 epitope [18,19]. In accordance with this suggestion, Wei et al. recently reported the expression of the HNK-1 epitope in GlcAT-I cDNA-transfected COS-7 and Lec 2 cells when cotransfected with the HNK-1 3-*O*-sulfotransferase [14]. Thus, in the cases of some specific developmental stages or under pathological conditions including cancers, GlcAT-I may participate in the synthesis of the HNK-1 epitope. In addition, considering that

the GlcAT-I cDNA-transfected COS-1 cells did not express any GlcAT-P activity using both asialoorosomucoid or *N*-acetyllactosamine in spite of the fact that the GlcAT-I cDNA-transfected cells exhibited over 10-fold higher expression of GlcAT-I activity than that of the mock-transfected cells (Table 2), the possibility that COS-1 cells contain an as yet unidentified acceptor substrate for GlcAT-I to form the HNK-1 epitope can not be ruled out. In future studies, it is necessary to determine the carbohydrate structure of the HNK-1 antibody-reactive product in COS-1 cells transfected with GlcAT-I cDNA.

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