Identification and Characterization of Three Drosophila melanogaster Glucuronyltransferases Responsible for the Synthesis of the Conserved Glycosaminoglycan-Protein Linkage Region of Proteoglycans

TWO NOVEL HOMOLOGS EXHIBIT BROAD SPECIFICITY TOWARD OLIGOSACCHARIDES FROM PROTEOGLYCANS, GLYCOPROTEINS, AND GLYCOSPHINGOLIPIDS*

Byung-Taek Kim†, Kazunori Tsuchida†, John Linececum§, Hiroshi Kitagawa†, Merton Bernfield‡,§, and Kazuyuki Sugahara†

From the †Department of Biochemistry, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658-8558, Japan and the §Departments of Pediatrics and Cell Biology, Harvard Medical School, Children's Hospital, Boston, Massachusetts 02115

The Drosophila melanogaster genome contains three putative glucuronyltransferases homologous to human GlcAT-I and GlcAT-P. These enzymes are predicted to be β1,3-glucuronyltransferases involved in the synthesis of the glycosaminoglycan (GAG)-protein linkage region of proteoglycans and the HNK-1 carbohydrate epitope of proteoglycans and the HNK-1 carbohydrate epitope of proteins, respectively. The genes encode active enzymes, which we have designated DmGlcAT-I, DmGlcAT-BSI, and DmGlcAT-BSII (where BS stands for “broad specificity”). Protein A-tagged truncated soluble cAT-BSI, and DmGlcAT-BSII (where BS stands for zymes, which we have designated DmGlcAT-I, DmGlcAT-BSI, and DmGlcAT-BSII act on a wide array of substrates with non-reducing terminal β1,3- and β1,4-linked Gal residues. Their highest activities are obtained with asialo-Gal1-3Gal1-4Xyl. Strikingly, DmGlcAT-I has specificity for Gal1-3Gal1-4Xyl, whereas DmGlcAT-BSI and DmGlcAT-BSII act on a wide array of substrates with non-reducing terminal β1,3- and β1,4-linked Gal residues. Their highest activities are obtained with asialoorosomucoid with a terminal Gal1-4GlcNAc sequence, indicating their possible involvement in the synthesis of the HNK-1 epitope in addition to the GAG-protein linkage region. Gal1-3GlcNAc and Gal1-3GalNAc, disaccharides widely found in N- and O-glycans of proteoglycans and glycolipids, also serve as acceptors for DmGlcAT-BSI and -BSII. Transcripts of all three enzymes are ubiquitously expressed throughout the developmental stages and in adult tissues of Drosophila. Thus, all three glucuronyltransferases are likely involved in the synthesis of the GAG-protein linkage region in Drosophila, and DmGlcAT-BSI and -BSII appear to be involved in various GlcUA transfer reactions for the synthesis of proteoglycans, glycoproteins, and glycolipids. This activity distinguishes these glucuronyltransferases from their mammalian homologs GlcAT-P and GlcAT-D (or -S). Sequence alignment of the Drosophila glucuronyltransferases with homologs in human, rat, and Caenorhabditis elegans demonstrates the conservation of a majority of the critical amino acid residues in the active sites of the three Drosophila enzymes.

Proteoglycans (PGs) play an essential role in a variety of biological processes such as cell-cell adhesion, cell proliferation, and tissue morphogenesis (1, 2). PGs consist of a core protein and sulfated glycosaminoglycan (GAG) side chains. PGs can be classified into three groups based on the nature of their GAGs: heparan sulfate (HS)-type PGs, chondroitin sulfate (CS)-type PGs, and keratan sulfate PGs. There is increasing evidence that uniquely sulfated domain structures of GAG side chains are critically involved in various functions of these PGs (3–5), and defective synthesis of GAGs causes aberrant morphology and even embryonic lethality during development (6).

In biosynthesis, HS or CS linear chains are differentially assembled on the common linkage region tetrasaccharide GlcUA1-3Gal1-3Gal1-4Xyl1-4, which takes place on a specific serine residue in a given core protein (7). Transfer of either α1,4-GlcNAc or β1,4-GalNAc to the tetrasaccharide linkage region terminus mediated by the GAG-specific hexosaminyltransferases determines and initiates HS (8–10) or CS assembly, respectively (Refs. 11–13; reviewed in Ref. 14). HS and CS chains are polymerized as relatively simple structures composed of repeating disaccharide units, -(4GlcNAco-1-GlcUA1-4GlcNAco-1-GlcUA1-4), by HS polymerases (15, 16) or chondroitin synthase (17), respectively. These structures are further modified by the cooperative action of specific epimerases and sulfotransferases to produce mature, biologically active chains (reviewed in Ref. 18).

The synthesis of the linkage region is initiated by the addition of a majority of the critical amino acid residues in the active sites of the three Drosophila enzymes. The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank† and EBI Data Bank with accession number(s) AB080695 (DmGlcAT-I), AB080696 (DmGlcAT-BSI), and AB080697 (DmGlcAT-BSII).

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† Deceased on March 18, 2002.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, Kobe Pharmaceutical University, 4-19-1 Motomakita-machi, Higashinada-ku, Kobe 658-8558, Japan. Tel.: 81-78-441-7570; Fax: 81-78-441-7569; E-mail: k-sugar@kobepharma-u.ac.jp.

§ The abbreviations used are: PG, proteoglycan; CS, chondroitin sulfate; GlcAT-I; D. melanogaster glucuronyltransferase I; DmGlcAT-BSI, D. melanogaster glucuronyltransferase with broad specificity I; DmGlcAT-BSII, D. melanogaster glucuronyltransferase with broad specificity II; GAG, glycosaminoglycan; GlcAT-I, glucuronyltransferase I; GlcAT-P, glucuronyltransferase P; GlcUA, D-glucuronic acid; HNK-1, heparan sulfate; RT, reverse transcriptase; MES, 2-(N-morpholino)ethanesulfonic acid; EST, expressed sequence tag.

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tion of a Xyl residue by xylosyltransferase (19) from UDP-Xyl to specific serine residues, followed by sequential additions of two Gal residues from UDP-Gal by galactosyltransferases I (20, 21) and II (22), respectively. The reaction is completed by a GlcUA transfer from UDP-GlcUA catalyzed by glucuronolactonase I (GlcAT-I) (reviewed in Ref. 4). GlcAT-I was the first member cloned by degenerate PCR using primers derived from the conserved domain sequences of glucuronolactonase P (GlcAT-P) (25) required for the addition of GlcUA to the terminal Galβ1-4GlcNAc sequence of glycoprotein oligosaccharides, producing the HKN-1 carbohydrate epitope precursor sequence GlcUAβ1-3Galβ1-4GlcNAc. The trisaccharide sequence then serves as the acceptor site for the 3-O-sulfotransferase (24, 25) that subsequently generates the HKN-1 epitope. Both GlcAT-I and GlcAT-P are β,3-glucuronyltransferases and share a high degree of homology. They form a unique gene family that apparently plays several critical roles during development. GlcAT-I is constitutively expressed in all tissues, being consistent with the wide distribution and a huge array of biological functions of GAGs (3). In contrast, GlcAT-P is specifically expressed in neural cells (26), possibly reflecting a specific role in neural development. Although it seems that in mammals, GlcAT-I and GlcAT-P play specific roles in the synthesis of PGs and glycoproteins, respectively, overexpression of GlcAT-P in Drosophila can produce the HKN-1 epitope in mammalian cells (27, 28). Therefore, it remains obscure whether there is some overlap between their specificities.

The presence of GAGs, including HS, CS, and nonsulfated chondroitin, have been demonstrated in the invertebrate organisms Drosophila melanogaster and Caenorhabditis elegans (29, 30). Furthermore, use of a conventional linkage region tetrasaccharide sequence was recently established for these invertebrate GAG chains (31), suggesting that their fundamental structures and biosynthetic mechanisms are similar to the mammalian GAG chains. A single synecon and two glypicans, all of which are HSPGs whose mammalian homologs function in vitro (23) required for the addition of GlcUA to the terminal Galβ1-4GlcNAc sequence, were recently isolated from Drosophila. The mammalian expression vector pEF-BOS/IP was generated by chemical characterization of the expressed enzymes. All three glucuronyltransferases, Galβ1-3Galβ1-4Xyl, N-acetyllactosamine, lactose, Galβ1-3GlcNAc, and Glβ1-3GalNAc were obtained from Sigma. N-Acetyllactosamine and lactose were purchased from Seikagaku Corp. (Tokyo, Japan). The probe Galβ1-3Galβ1-4Xyl was a gift from Dr. Nancy B. Schwartz (University of Chicago, Chicago, IL). Asialoorosomucoid was prepared as described previously (40). β-Glucuronidase (EC 3.2.1.31), homogeneously purified from Ampullaria (freshwater apple shell) hepatopancreas, was obtained from Tokyo Zouki Chemical Co. (Tokyo, Japan). A Superoxide™ Peptide HR1030 column and IgG-Sepharose were obtained from Amersham Biosciences.

**Data Base Search for GlcAT-I Homologs**—The amino acid sequence of human GlcAT-I was used for BLAST search of GenBank™ and the homepage of the Berkeley Drosophila Genome Project (www.fruitfly.org), which identified three putative glucuronyltransferases. Their GenBank™ accession numbers are CAA21824, AAF50082 (the CG6207 clone), and AAF52795 (the CG3881 clone). In order to obtain homology higher to mammalian GlcAT-I, which were tentatively named CAA21824, CG6207, and CG3881. The corresponding Drosophila EST clones were purchased from Research Genetics, Inc. (Huntsville, AL).

**Construction of Soluble Forms of the Three Putative Glucuronyltransferases**—The mammalian expression vector pEF-BOS/IP was generated as described previously (10). The truncated form of CAA21824, lacking the amino-terminal 27 amino acids, was amplified by PCR with the cDNA from the Drosophila EST clone GH05057 using a 5′-primer (5′-CGGGATCCACGGCGAGCTCCTTGCTAAT-3′) containing an in-frame BamHI site and a 3′-primer (5′-CCGGATCTCATCCTGCTCATGCGGC-3′) containing a BamHI site just after the stop codon. The amplification of the first 61 amino acids of the truncated form was amplified with the obtained cDNA from the GH22332 clone as a template using a 5′-primer (5′-ATGATCCGCTTACATATGCGAGGATT-3′) containing an in-frame BamHI site and a 3′-primer (5′-ATGATCCTGCGATCCGAGATTGT-3′) containing a BamHI site located 59 base pairs downstream of the stop codon. The soluble form of CG6207, lacking the first 59 amino acids at the amino-terminal end, was amplified with the plasmids from RE26967 using a 5′-primer (5′-CGGGATCTTCCGCGGACCCAGGATGTTT-3′) containing an in-frame BamHI site and a 3′-primer (5′-CGGGATCCACGGCGAGCTCATGCTCTTAC-3′) containing a BamHI site at 28 base pairs downstream of the stop codon. All the polymerase chain reactions were carried out using Pfu polymerase (Stratagene). The amplified fragments were digested with BamHI and cloned into the BamHI site of pEF-BOS/IP, resulting in fusion proteins with a cleavable insulin signal sequence for secretion and protein A for purification of the expressed fusion proteins.

**Expression of the Soluble Forms of Three Glucuronyltransferases and Enzyme Assays**—The respective expression vector (6.7 µg) was introduced into COS-1 cells using FuGENE™6 (Roche Molecular Biochemicals) according to the provided instructions. The transfected cells were incubated at 30 °C for 48 h, and a 1 ml aliquot of the culture medium was used for incubation with 10 µl of an IgG-Sepharose suspension for 2 h at 4 °C. The enzyme-bound beads were recovered by centrifugation, washed with and suspended in the assay buffer for enzyme assay. A glucuronolactonase assay mixture contained 50 mM MES-NaOH buffer, pH 6.5, 171 µM ATP, 10 mM MnCl₂, 14.3 µM UDP-[14C]GlcUA (1.1 × 10⁵ dpm), and 1 nmol of each acceptor in a total volume of 30 µl. The tested acceptors were Galβ1-3Galβ1-4Xyl, N-acetyllactosamine, lactose, Galβ1-3GlcNAc, Galβ1-3GalNAc, and asialoorosomucoid (Galβ1-4GlcNAc-R, where R represents the remainder of the N-linked oligosaccharide chain). All the reaction mixtures were incubated at 25 or 37 °C. The radiolabeled product obtained with asialoorosomucoid was separated from the donor UDP-[14C]GlcUA by gel filtration on a Superdex™ Peptide column. The isolation of the [14C]-labeled products obtained with the other acceptor substrates was carried out by applying the reaction mixture to Pasteur pipet tips followed by ultrafiltration (Millipore, 300 kDa, 100 µm mesh) and quantification in a liquid scintillation counter (TRI-CARB 2900TR, Packard Co.) using a scintillation fluid containing 1.2% (w/v) 2,5-diphenyloxazole and 33% (w/v) Triton X-100.

**Characterization of the Glucuronyl Transferase Reaction Products**—The reaction products obtained with two different acceptors, Galβ1-3Galβ1-4Xyl and asialoorosomucoid, were characterized by β-glucuronidase digestion. Isolation of the products from the reaction mixtures was carried out as described above. The individual isolated products were digested with 20 mililiters of Ampullaria β-glucuronidase for 2 h at 37 °C overnight and then analyzed by gel-filtration chromatography on a Superdex™ Peptide column.

**Determination of Expression Levels of Three Glucuronyltransferases by Semiquantitative RT–PCR**—A Drosophila Rapid-Scan™ gene ex-
pression panel purchased from OriGene Technologies, Inc. (Rockville, MD) provides a semiquantitative method for determining the Drosophila gene expression level. The panel contained first strand cDNAs from different Drosophila tissues and developmental stages, which have been normalized against the RP49 transcript, the mRNA encoding a constitutively expressed ribosomal protein. The following primer pairs for each gene were used to amplify DNA fragments of 639, 497, and 715 bp, respectively: a 5'-primer (5'-AGAACTTGACG-3') and a 3'-primer (5'-CGGTTTTTACCAACTGCC-3') for CAA21824, a 5'-primer (5'-GGTGTAGCTCTTACGGATCTGAG-3') and a 3'-primer (5'-AGCGGACCTGATGCGAAG-3') for CG3881, and a 5'-primer (5'-ATGAAGGGCCGAACTACC-3') and a 3'-primer (5'-AGAATTGACGCTTGATGAG-3') for CG6207. PCR was carried out, with a 15 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s.

RESULTS

Identification of Drosophila Homologs of Human GlcAT-I—BLASTP analysis of the D. melanogaster genome, using the amino acid sequence of human GlcAT-I, identified three putative glucuronyltransferases (CAA21824, CG6207, and CG3881). These three predicted proteins consisted of 313, 479, and 366 residues with an NH2-terminal cytoplasmic tail of 9 amino acid residues, respectively: a 5'-primer (5'-CGGTTTTTACCAACTGCC-3') and a 3'-primer (5'-AGAACTTGACG-3') for CAA21824, a 5'-primer (5'-GGTGTAGCTCTTACGGATCTGAG-3') and a 3'-primer (5'-AGCGGACCTGATGCGAAG-3') for CG3881, and a 5'-primer (5'-ATGAAGGGCCGAACTACC-3') and a 3'-primer (5'-AGAATTGACGCTTGATGAG-3') for CG6207. PCR was carried out, with a 15 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s.

The complete nucleotide sequence of DmGlcAT-I cDNA and the predicted amino acid sequence of DmGlcAT-I are shown. The putative transmembrane domain and polyadenylation signal are underlined. A potential N-glycosylation site is marked by asterisk.

Fig. 1. Structure of DmGlcAT-I cDNA. The complete nucleotide sequence of DmGlcAT-I cDNA and the predicted amino acid sequence of DmGlcAT-I are shown. The putative transmembrane domain and polyadenylation signal are underlined. A potential N-glycosylation site is marked by asterisk.

glycosyltransferases. The three putative glucuronyltransferases of the corrected sequences derived from CAA21824, CG3881, and CG6207 were designated DmGlcAT-I, DmGlcAT-BSI, and DmGlcAT-BSII, respectively, based on their homology to mammalian GlcAT-I and the broad specificity for the acceptor recognition of the others (see below).

The cDNA of DmGlcAT-I, derived from EST clone GH05057, consisted of an open reading frame encoding 306 amino acid residues with an NH2-terminal cytoplasmic tail of 9 residues, a putative transmembrane domain of 17 residues, and one potential N-glycosylation site (Fig. 1). The cDNA of DmGlcAT-BSI derived from EST clone GH22332 was predicted to encode 366 residues with an NH2-terminal cytoplasmic tail of 9 amino acid residues and a putative transmembrane domain of 22 amino acid residues and three potential N-glycosylation sites (Fig. 2). The DmGlcAT-BSII cDNA was obtained from EST clone EMy967 and encoded 316 amino acid residues with a relatively long NH2-terminal cytoplasmic tail of 35 residues, a putative transmembrane domain of 15 residues, and two potential N-glycosylation sites, although another potential initiation codon predicted a 9-residue cytoplasmic tail (Fig. 3).

Using the ClustalW alignment method from the DNA Data Bank of Japan (DDBJ) homepage, multiple sequence alignment for the reported glucuronyltransferases was performed. Fig. 4A shows the aligned sequences of the three Drosophila proteins and the glucuronyltransferases from human, rat, and C. elegans. SQV-8 is orthologous to C. elegans GlcAT-I and possesses a functional enzyme activity (34, 45). DmGlcAT-I, DmGlcAT-BSI, and DmGlcAT-BSII showed 36–30, and 27–30% amino acid identities, respectively, to mammalian glucuronyltransferases and 24–35% amino acid identity to one another. Interestingly, DmGlcAT-I showed much higher homology (39%) to SQV-8 (C. elegans GlcAT-I) than the other two homologs: CG6207 (27%) and CG3881 (25%), suggesting that DmGlcAT-I might be a GlcAT-I ortholog in Drosophila. This idea was also supported by the phylogenetic tree generated using the whole amino acid sequences of these proteins, in which DmGlcAT-I was grouped together with human GlcAT-I, whereas Dm-
GlcAT-BSI and DmGlcAT-BSII were separately categorized (Fig. 4B).

**All Three Proteins Possess Glucuronyltransferase Activities**—To determine whether the three predicted proteins are functional glucuronyltransferases, their putative catalytic domains were expressed as chimeric proteins fused to an IgG-binding domain of bacterial protein A. The secreted proteins were purified with IgG-Sepharose beads to eliminate endogenous glycosyltransferases, and the enzyme-bound beads were subjected to glucuronyltransferase assays.

When the linkage trisaccharide Gal\(^{1} \rightarrow 3\)Gal\(^{1} \rightarrow 4\)Xyl, an authentic substrate for GlcAT-I, was used as an acceptor and UDP-GlcUA as a donor substrate, significant GlcUA transferase activities were detected for all three proteins (Table I). Although DmGlcAT-I showed a relatively low efficiency compared with DmGlcAT-BSI and DmGlcAT-BSII, it did not utilize any other acceptor substrates, showing strict acceptor specificity toward Gal\(^{1} \rightarrow 3\)Gal\(^{1} \rightarrow 4\)Xyl. In this regard, DmGlcAT-I resembled human GlcAT-I. Interestingly, unlike the other two enzymes, DmGlcAT-I showed no detectable GlcAT-I activity at 37 °C. Accordingly, although DmGlcAT-BSI and DmGlcAT-BSII showed their highest activities at 37 °C, all the enzyme activities were measured at 25 °C for comparison, which would be closer to the body temperature of *Drosophila*.

DmGlcAT-BSI and DmGlcAT-BSII transferred GlcUA from UDP-GlcUA not only to Gal\(^{1} \rightarrow 3\)Gal\(^{1} \rightarrow 4\)Xyl but also to N-acetyllactosamine (Gal\(^{1} \rightarrow 4\)GlcNAc) and asialoorosomucoid (Gal\(^{1} \rightarrow 4\)GlcNAc-R) (Table I). These reactions are considered to represent the glucuronyltransferase reaction involved in the synthesis of the nonsulfated precursor for the 3-O-sulfated GlcUA-containing HNK-1 carbohydrate epitope on glycoproteins (28). In addition, DmGlcAT-BSI and DmGlcAT-BSII could utilize Galβ1–3GalNac, Galβ1–4GlcNac, and lactose (Galβ1–4Glc), with the highest activity toward Galβ1–3GalNac. The trisaccharide sequence GlcUAβ1–3GalNAc of one of these products has been reported for glycosphingolipids in three dipterans including *D. melanogaster* (48–50), indicating that the detected glucuronyltransferase activity is involved in the synthesis of acidic glycosphingolipids as well. Trisaccharide sequences of the reaction products, GlcUAβ1–3GalNAc and GlcUAβ1–3Galβ1–4Glc, have not been reported so far. These findings indicate that DmGlcAT-BSI and DmGlcAT-BSII have a broad spectrum in their substrate specificity, which clearly distinguishes these enzymes from DmGlcAT-I. It should be noted that none of the three glucuronyltransferases transferred GlcUA from UDP-GlcUA to either N-acetylhexasosan oligosaccharides with terminal GlcNAc (16) or chondroitin (data not shown), excluding the possibility that they might be involved in the synthesis of the repeating disaccharide regions of HS or CS chains. The medium recovered from the COS-1 cells transfected with the empty vector showed no glucuronyltransferase activity, confirming that all the observed activities are attributable to the expressed recombinant enzymes.

**Characterization of the Anomeric Configuration of the Glucuronyl Linkages Produced by Drosophila Glucuronyltransferase Reactions**—The anomeric configuration of the GlcUA residues in the transferase reaction products was characterized by β-glucuronidase digestion. The GlcAT-I reaction products obtained using Galβ1–3Galβ1–4Xyl as an acceptor and each of the three glucuronyltransferases and the GlcAT-P reaction products obtained using asialoorosomucoid as an acceptor and
each of the two glucuronyltransferases (DmGlcAT-BSI and DmGlcAT-BSII) were separated from the unutilized UDP-[14C]GlcUA as described under "Experimental Procedures." Ten pmol each of the reaction product was digested with Am-
pullaria/H9252-glucuronidase and analyzed by gel filtration chro-
matography on a Superdex™ Peptide column. The radiolabels
of both GlcAT-I and GlcAT-P reaction products were completely
released by/H9252-glucuronidase digestion, resulting in the shift of
the radioactive peak to the free GlcUA position (Figs. 5 and 6),
suggesting that the GlcUA residues had been transferred to the
non-reducing termini of the respective acceptor substrates by
all three enzymes through/H9252-configuration.

Expression Profile of Three Drosophila Glucuronyltrans-
ferases—To investigate spatiotemporal expression of Dm-
GlcAT-I, DmGlcAT-BSI, and DmGlcAT-BSII, their expression
profile was determined by RT-PCR using a commercial cDNA
panel, in which first strand cDNAs from different tissues and
developmental stages of Drosophila were serially diluted over a
4-log range with the highest concentration (H11003 1000) correspond-
ing to 1 ng of cDNA in a PCR-ready tube. Although this method
is semiquantitative, it was possible to grossly compare expres-
sion patterns of three Drosophila genes. The results obtained
from the highest cDNA concentration only are displayed in Fig.
7 because the amplified bands observed below this concentra-
tion were faint for all three genes. The transcripts of all three
glucuronyltransferases were ubiquitously expressed; a meas-
urable amount of expression was detected even in the earliest
embryonic stage (0–4-h embryo) for all enzymes. In particular,
DmGlcAT-BSI and DmGlcAT-BSII transcripts were expressed
more strongly as compared with that of DmGlcAT-I, which may
suggest their relative importance during embryonic develop-
ment of Drosophila.

DISCUSSION
In this study, we have identified three putative glucuronyl-
transferases in the D. melanogaster genome and demonstrated
that they are catalytically active enzymes likely involved in the
biosynthesis of various glycoconjugates. DmGlcAT-I showed
strict acceptor substrate specificity toward the trisaccharide
Gal/H9252 1–3Gal/H9252 1–4Xyl derived from the protein linkage region of
GAGs, as in the case of human GlcAT-I (27). It is noteworthy
that SQV-8, GlcAT-I ortholog in C. elegans, shows significant
activities toward several artificial substrates representing
sugar sequences found in glycoproteins (34). These different
substrate specificities of the two GlcAT-I orthologs suggest that
DmGlcAT-I may have diverged earlier than SQV-8 in the evo-
lationary tree, which can also be expected from the phyloge-
netic tree shown in Fig. 4 B.

In contrast to DmGlcAT-I, DmGlcAT-BSI and DmGlcAT-
BSII showed broad acceptor specificity, utilizing all tested ac-
ceptor substrates (Table I). Interestingly, both enzymes were
potently active toward the linkage region trisaccharide
Galβ1-3Galβ1-4Xyl, showing ~2-fold greater activity than Dm-
GlcAT-I. However, their highest activity was obtained with
asialoorosomucoid (Table I), an acceptor substrate for GlcAT-P
involved in the synthesis of the HNK-1 carbohydrate epitope
precursor oligosaccharide on glycoproteins. The HNK-1 epitope
is exclusively found on membrane-bound cell recognition mol-
ecules in nervous tissues of vertebrates and is involved in
cell-cell and cell-matrix adhesion. Recent studies showed that
the HNK-1 carbohydrate antigen is also present in insects
including D. melanogaster (40, 49–51). As in the case of Dro-
sophila, two kinds of HNK-1 epitope-synthesizing glucuronyl-
transferases have been described for mammals: GlcAT-P and

FIG. 3. Structure of DmGlcAT-BSII cDNA. The complete nucleotide sequence of DmGlcAT-BSII cDNA and the predicted amino acid sequence
of DmGlcAT-BSII are shown. The presumptive two start codons, putative transmembrane domain, and polyadenylation signal are underlined. Two potential N-glycosylation sites are marked by asterisks.
GlcAT-D (or GlcAT-S), both of which were initially thought to be specific for glycoprotein oligosaccharides (26, 43), but were subsequently shown to act on glycolipids such as paragloboside as well (41, 44). However, if compared with DmGlcAT-BSI and DmGlcAT-BSII, these mammalian enzymes with dual acceptor specificity still have the rigid substrate specificity in that neither utilizes lactose (27), and GlcAT-P does not act on the linkage region trisaccharide Gal¹⁻³Gal¹⁻⁴Xyl (27).

**Fig. 4.** Comparison of three putative Drosophila glucuronyltransferases with the reported glucuronyltransferases involved in synthesis of either the GAG-protein linkage region or the HNK-1 carbohydrate epitope. A, multiple sequence alignment of DmGlcAT-I, DmGlcAT-BSI and DmGlcAT-BSII with the glucuronyltransferases reported to date among human (23, 26), rat (43, 44), and C. elegans (34, 45). Introduced gaps are shown by hyphens. Closed boxes indicate identical amino acids in all proteins. Amino acids identical in more than four proteins are shaded. The conserved DXXD motifs are underlined. The amino acid residues that interact with uracil and GlcUA, are indicated by asterisks and closed circles, respectively (46, 47). Arrows indicate the amino acids interacting with non-reducing terminal Gal of the acceptor trisaccharide Gal¹⁻³Gal¹⁻⁴Xyl (46). Glu marked with closed square is a catalytic base (46). Arg marked by open circle interacts with β-phosphate of UDP (47). Arg marked by triangle interacts with 6-hydroxyl group of the terminal Gal of the acceptor (46). B, the phylogenetic tree based on the above alignment. The multiple sequence alignment and the phylogenetic tree were produced using ClustalW. hGlcAT-I, human GlcAT-I; hGlcAT-P, human GlcAT-P; rGlcAT-D, rat GlcAT-D.
Three Drosophila Glucuronyltransferases

Comparison of the acceptor substrate specificity of truncated forms of the three Drosophila glucuronyltransferases

| Acceptor                          | Activitya | DmGlcAT-I | DmGlcAT-BSI | DmGlcAT-BSII |
|-----------------------------------|-----------|-----------|-------------|-------------|
| Galβ1-3Galβ1-4Xyl                 | 18.6 (ND) | 34.4 (117)| 32.5 (128)  |
| Asialoorosomucoid                 | ND (ND)   | 72.5 (227)| 61.4 (292)  |
| N-Acetyllactosamine               | ND (ND)   | 3.4 (21)  | 23.7 (107)  |
| Lactose                          | ND (ND)   | 4.6 (16)  | 9.0 (64)    |
| Galβ1-3GlcNAc                     | ND (ND)   | 39.6 (141)| 15.4 (55)   |
| Galβ1-3GlcNac                     | ND (ND)   | 44.5 (154)| 21.0 (91)   |

a The enzyme assays were carried out at 25 or 37 °C as described under “Experimental Procedures,” and the enzyme activities measured at 37 °C are shown in parentheses. The values represent the average of two independent experiments.

β Galβ1-4GlcNAc-R, where R represents the remainder of the N-linked oligosaccharide chain.

γ Galβ1-4Glc.

δ ND, not detected (<0.5 pmol/ml medium/h).

Fig. 5. Characterization of GlcAT-I reaction products obtained with Galβ1-3Galβ1-4Xyl as acceptor and DmGlcAT-I, DmGlcAT-BSI, and DmGlcAT-BSII as enzyme proteins. Each GlcAT-I reaction product obtained with Galβ1-3Galβ1-4Xyl by DmGlcAT-I (A), DmGlcAT-BSI (B), and DmGlcAT-BSII (C) was digested with β-glucuronidase (closed square), and each digest or an undigested sample (open circle) was gel-filtrated on a Superdex™ Peptide HR 10/30 column as described under “Experimental Procedures.” The separated fractions (0.4 ml each) were measured for radioactivity. An arrow indicates the elution position of free GlcUA, and a closed arrowhead and an open arrowhead show the void volume and the total volume of the column, respectively.

In contrast, DmGlcAT-BSI and DmGlcAT-BSII exhibited broader acceptor specificities than these mammalian enzymes as discussed below, utilizing all tested acceptor oligosaccharide substrates containing terminal β1,3- and β1,4-linked Gal residues, which are found in glycoproteins, glycolipids, or PGs (Table I).

DmGlcAT-BSI and DmGlcAT-BSII transferred GlcUA to Galβ1-3Galβ1-4GlcNAc (Table I), producing the terminal sequence GlcUAβ1-3Galβ1-3GlcNAc of the acidic glycosphingolipids reported for dipterans including Drosophila (48–50). Interestingly, such glycosphingolipids were shown in larvae and in the head of adult females of Drosophila, where the carbohydrate epitope was detected in non-neural as well as neural tissues (40). The same trisaccharide sequence has been reported for mucin-type O-glycans in C. elegans as well (51), although no such glycosphingolipids or mucin-type oligosaccharides that contain GlcUA have been reported for vertebrates. The trisaccharide sequences, GlcUAβ1-3Galβ1-3GlcNAc and GlcUAβ1-3Galβ1-4Glc, of the glucuronyltransfer reaction products observed in this study (Table I), have not previously been reported in other organisms. However, a glucuronyltransfer reaction to lactose was previously reported for cell-free extracts from embryonic chick cartilage (52), which may indicate the existence of such glycoconjugates in vertebrates. Although GlcUAβ1-3Galβ1-3GlcNAc structure has not been demonstrated for any organisms, the revealed unequivocal enzyme
activity toward Galβ1–3GlcNac, which is a part of widespread type 1 glycan chains, may suggest the existence of its glucuronolylated products at least in Drosophila. Considering the catalytic activities toward various types of acceptor oligosaccharides and ubiquitous expression of DmGlcAT-BSI and DmGlcAT-BSII throughout the developmental stages and in adult tissues, it is speculated that their catalytic activities have pivotal roles in forming diverse GlcUA-containing glycoconjugates including GAGs, N- and O-linked glycoproteins, and glycolipids in Drosophila, and are involved in neuronal events. Comprehensive and vigorous analysis of chemical structure of various types of glycoconjugates in Drosophila is required to understand in depth biological functions of these glycosyltransferases.

The recently solved crystal structure of human GlcAT-I revealed the essential amino acid residues in the active site (46, 47), which are well conserved in Drosophila glucuronyltransferases. The five amino acid residues (marked by closed circles) corresponding to Asp\textsuperscript{194}, Arg\textsuperscript{199}, Arg\textsuperscript{206}, Asp\textsuperscript{252}, and His\textsuperscript{308} in human GlcAT-I, which interact with the donor GlcUA, are completely conserved in all aligned sequences (Fig. 4A). Also important are the four residues corresponding to Glu\textsuperscript{227}, Arg\textsuperscript{247}, Asp\textsuperscript{252}, and Glu\textsuperscript{281} of human GlcAT-I, which interact with the acceptor sugar (marked by arrows) (46). Among these, completely conserved are the Glu at the corresponding position of Glu\textsuperscript{231} of human GlcAT-I (marked by closed square), which acts as a catalytic base (46), as well as two other residues corresponding to Arg\textsuperscript{247} and Asp\textsuperscript{252} which are involved in recognition of the non-reducing terminal Gal of Gal(1–3Gal)1–4Xyl (46, 47). The catalytic Glu residue activates the C3 hydroxyl group of the acceptor sugar (Gal) for nucleophilic attack at C1 by deprotonation. Thus, the major amino acids in the active site have been maintained irrespective of species. Taken together, the findings in the present study strongly suggest that the GlcUA transferase reaction of the three Drosophila glucuronyltransferases proceeds in the same inversion mechanism (47) among all the enzymes. Three amino acid residues in the conserved domains have been replaced by other residues in DmGlcAT-BSI and DmGlcAT-BSII. The Arg\textsuperscript{210} of human GlcAT-I (marked by triangle), which interacts with a β-phosphate of UDP (47), has been replaced by Gln in DmGlcAT-BSI and DmGlcAT-BSII. However, the other residues interacting directly (Asp\textsuperscript{252}) or indirectly (Asp\textsuperscript{194}, Asp\textsuperscript{196}, and Asn\textsuperscript{197}) through manganese ion and a water molecule with the β-phosphate are well conserved. In the acceptor-interacting regions of DmGlcAT-BSI and DmGlcAT-BSII, both enzymes had Ser in place of conserved Glu (Glu\textsuperscript{227} in human GlcAT-I). Because this Glu (marked by arrow and open circle) appears to interact with 6-hydroxyl of the terminal Gal (46), it is speculated that this change from Glu to Ser might be responsible for their unique substrate tolerance. In COOH-terminal region, one Cys residue is conserved in all enzymes (Fig. 4A, double-underlined), and replacement of this Cys with Ala totally inactivates human GlcAT-I; therefore, this amino acid residue has been suggested to exist in the catalytic site (53). Because, however, crystallographic data indicate that this Cys residue does not exist in the vicinity of the catalytic sites (46, 47), the role of this Cys remains controversial.

All three Drosophila glucuronyltransferases exhibited evident transferase activity toward the linkage region trisaccharide, indicating that GAG biosynthesis in Drosophila appear to be regulated at the level of the linkage region differently from those of mammalian systems. However, it remains to be investigated how these three enzymes play their individual roles in terms of the synthesis of the GAG-protein linkage region of different types of GAG chains or different types of PGs in various tissues and during development. Comprehensive and thorough analysis of glycoconjugates of mutants deficient in each one of these enzymes or double mutants would also be required for better understanding of functional roles of these enzymes in GAG biosynthesis.

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Identification and Characterization of Three *Drosophila melanogaster* Glucuronyltransferases Responsible for the Synthesis of the Conserved Glycosaminoglycan-Protein Linkage Region of Proteoglycans: TWO NOVEL HOMOLOGS EXHIBIT BROAD SPECIFICITY TOWARD OLIGOSACCHARIDES FROM PROTEOGLYCANS, GLYCOPROTEINS, AND GLYCOSPHINGOLIPIDS

Byung-Taek Kim, Kazunori Tsuchida, John Lincecum, Hiroshi Kitagawa, Merton Bernfield and Kazuyuki Sugahara

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