Phosphorylation and sulfation of oligosaccharide substrates critically influence the activity of human β1,4-galactosyltransferase 7 (GalT-I) and β1,3-glucuronosyltransferase I (GlcAT-I) involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans

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Running title: Substrate specificity of human GalT-I and GlcAT-I
We determined whether the two major structural modifications, i.e., phosphorylation and sulfation, of the glycosaminoglycan-protein linkage region (GlcA\(\beta\)1-3Gal\(\beta\)1-3Gal\(\beta\)1-4Xyl\(\beta\)1) govern the specificity of the glycosyltransferases responsible for the biosynthesis of the tetrasaccharide primer. We analyzed the influence of C-2 phosphorylation of Xyl residue on human \(\beta\)1,4-galactosyltransferase 7 (GalT-I), which catalyzes the transfer of Gal onto Xyl and we evaluated the consequences of C-4/C-6 sulfation of Gal\(\beta\)1-3Gal (Gal2-Gal1) on activity and specificity of \(\beta\)1,3-glucuronosyltransferase I (GlcAT-I) responsible for the completion of the glycosaminoglycan primer sequence. For this purpose, a series of phosphorylated xylosides and sulfated C-4 and C-6 analogs of Gal\(\beta\)1-3Gal was synthesized and tested as potential substrates for the recombinant enzymes. Our results revealed that phosphorylation of Xyl on C-2 position prevents GalT-I activity, suggesting that this modification may occur once Gal is attached to the Xyl residue of the nascent oligosaccharide linkage. On the other hand, we showed that sulfation on C-6 position of Gal1 of the Gal\(\beta\)1-3Gal analog markedly enhanced GlcAT-I catalytic efficiency and we demonstrated the importance of W243 and K317 residues of Gal1 binding site for enzyme activity. In contrast, we found that GlcAT-I was unable to use digalactosides as acceptor substrates when Gal1 was sulfated on C-4 position or when Gal2 was sulfated on both C-4 and C-6 positions. Altogether, we demonstrated that oligosaccharide modifications of the linkage region control the specificity of the glycosyltransferases, a process which may regulate maturation and processing of GAG chains.
Proteoglycans (PGs), which are located at cell surfaces and in extracellular matrix play vital functions in many biological processes such as cell proliferation, cell adhesion, blood coagulation and wound repair (1). In most cases, biological activities of PGs are governed by interactions of their glycosaminoglycan (GAG) chains with growth factors, cytokines, morphogens and a variety of protein ligands. Accordingly, disruption of the glycosyltransferases involved in either initiation or elongation of GAG chains has severe biological consequences. In human, mutations of the gene encoding \( \beta 1,4 \)-galactosyltransferase 7 (GalT-I) cause a progeroid variant of Ehlers-Danlos syndrome characterized by aged appearance, developmental delay, dwarfism and various connective abnormalities (2). Likewise, it has been shown that mutations of \( EXT1 \) and \( EXT2 \) genes coding for glycosyltransferases responsible for heparan-sulfate (HS) chains polymerization lead to the hereditary multiple exostoses disorder, characterized by tumors of bony outgrowth (3).

The major GAGs found on PGs are HS/heparin and chondroitin-sulfate (CS)/dermatan-sulfate, which are linear polysaccharides consisting of repetition of \([\text{GlcNAc} \alpha 1-4\text{GlcA} \beta 1-4] \) and \([\text{GalNAc} \beta 1-4\text{GlcA} \beta 1-3] \) disaccharide units, respectively. The assembly of GAG chains is initiated by the synthesis of a common GAG-protein linkage structure \( \text{GlcA} \beta 1-3\text{Gal} \beta 1-3\text{Gal} \beta 1-4\text{Xyl-O-} \), which is attached to specific serine residues of different core proteins. This linkage tetrasaccharide is formed by the stepwise addition of each sugar residue, from the corresponding UDP-sugar, catalyzed by \( O \)-xylosyltransferase I (4), GalT-I (5), \( \beta 1,3 \)-galactosyltransferase 6 (GalT-II) (6) and \( \beta 1,3 \)-glucuronosyltransferase I (GlcAT-I) (7) (see Fig. 1A). The transfer of either an \( \alpha \text{GlcNAc} \) or \( \beta \text{GalNAc} \) residue on the terminal GlcA of the linkage region initiates the polymerization of HS or CS chains, respectively. HS and CS are further modified by the cooperative action of epimerases and sulfotransferases which adds considerable complexity and functionality to the polysaccharide GAG chains.

Recently, structural studies showed the presence of various modifications of the GAG-protein linkage region (see for review ref 8). Sulfate substitutions are primarily found at \( C-4 \) and/or \( C-6 \) position of the outer Gal (referred as Gal2) and of the inner Gal (referred as Gal1) of the linkage tetrasaccharide, whereas the presence of a phosphate has been exclusively demonstrated on the \( C-2 \) position of Xyl (see Fig. 1A). \( C-6 \) sulfation of Gal2 has been identified as the predominant modification in human aggrecan CS chains (9) and \( C-4 \) sulfation of the same Gal residue has been
found in urinary trypsin inhibitor (10) and inter-α-trypsin inhibitor (11). Sulfation at 4- and 6-positions of Gal1 and/or Gal2 is also present in CS from aggrecan of shark cartilage (12), bovine nasal septum (13), articular bovine cartilage (14) and from mouse syndecan-1 (15). So far, sulfated Gal residues of the linkage region have been demonstrated for CS and DS but not in HS or heparin, whereas a C-2 phosphorylated Xyl residue has been found in both HS/heparin and CS/DS chains. Although the role of these substitutions is not fully understood, it has been suggested that phosphorylation and sulfation may regulate maturation and processing of growing GAG chains (16, 17). A prerequisite step in the evaluation of this hypothesis is the determination of the substrate specificity of the glycosyltransferases responsible for the biosynthesis of the linkage region with regard to phosphorylation and sulfation. Our group has been deeply involved in the structure-function studies of GlcAT-I, which catalyzes the transfer of GlcA onto Gal2 of Galβ1-3Gal, thus completing the final step of GAG-protein linkage synthesis (18-20). This enzyme has attracted much attention in our lab and others (21) since it appears to play a rate-limiting role in GAG biosynthesis (22, 23). GalT-I, which is responsible for the transfer of Gal onto the Xyl residue of the glycopeptide primer of PGs, has recently been cloned but has not been deeply characterized (5).

In this study, we determined whether phosphorylation of Xyl and sulfation of Gal1 and/or Gal2 affects GalT-I and GlcAT-I activity, respectively. For this purpose, we designed and synthesized C-2 phosphorylated xylosides as well as C-4 and/or C-6 sulfated digalactose analogs of the GAG-protein linkage region. These compounds have been tested as potential substrates of the two recombinant human enzymes expressed in the yeast Pichia pastoris (P. pastoris). We showed that GalT-I efficiently catalyzed the transfer of Gal onto the nonphosphorylated xyloside whereas the phosphorylated analog was not substrate, suggesting that C-2 phosphorylation precludes the transfer of the first Gal on Xyl of the glycopeptide primer. By constrast, we demonstrated that the Galβ1-3Gal(6-sulfate) derivative serves as a much better acceptor substrate for the recombinant human GlcAT-I than its unsulfated counterpart, raising the possibility that sulfation at the C-6 position of Gal1 may contribute to the efficient completion of the linkage region tetrasaccharide. Our data show that phosphorylation and sulfation critically influence the specificity of the glycosyltransferases involved in the formation of the GAG-protein linkage region.
EXPERIMENTAL PROCEDURES

Materials - UDP-Gal, UDP-GlcA, anti-rabbit alkaline phosphatase-conjugated immunoglobulins were from Sigma (L’Isle d’Abeau, St. Quentin Fallavier, France). The products used for chemical synthesis were provided from Aldrich (L’Isle d’Abeau, St. Quentin Fallavier, France). UDP[\(^{14}\)C]-GlcA and UDP[\(^{14}\)C]-Gal were purchased from Amersham Biosciences (Saclay, France). Trifluoroacetic acid and dimethylsulfoxide were obtained from Merck (Darmstadt, Germany) and HPLC grade acetonitrile from Carlo Erba (Val de Reuil, France). Bacterial and yeast culture media were provided by Difco Becton Dickinson (Le Pont le Chaix, France). Protein assay reagent was obtained from Bio-Rad (Hercules, CA). Restriction enzymes, T4 DNA ligase were provided by New England Biolabs (Hitchin, UK). The \textit{P. pastoris} expression system and competent \textit{Escherichia coli} cells were purchased from Invitrogen (Groningen, The Netherlands). The QuickChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA).

Chemical synthesis – The 7-methoxy-2-naphthyl (MN)-derivated xylosides, in which the methoxy group served as a marker for NMR characterization and UV detection were prepared as described below. The structure of the compounds is presented in Fig. 1B: Xyl1\(-\text{O-MN}\) (1), Xyl(2-phosphate)\(\beta\)1\(-\text{O-MN}\) (2), Gal\(\beta\)1\(-\text{4Xyl}\)\(\beta\)1\(-\text{O-MN}\) (3), Gal\(\beta\)1\(-\text{4Xyl}(2\text{-phosphate})\)\(\beta\)1\(-\text{O-MN}\) (4). Briefly, treatment of known D-Xyl peracetate with 7-methoxy-2-naphthol in dichloromethane under the catalysis of trimethylsilyl triflate gave the crystalline (m.p. 154-155°C) \(\beta\)-linked glycoside in 77% yield. Transesterification of the latter compound with sodium methoxide in methanol gave quantitatively the Xyl\(\beta\)1\(-\text{O-MN}\) derivative (m.p. 176-177°C). Treatment of this triol with 2-methoxypropene and (+,-)camphorsulfonic acid in \(N,N\)-dimethylformamide under kinetic control afforded the 2,3\(-\text{O-isopropylidene derivative}\) (m.p. 137-138°C) and its 3,4-isomer (m.p. 183-184°C) in 69 and 18% yields, respectively. Phosphorylation at \(O\)-2 was achieved through treatment of the 3,4\(-\text{O-isopropylidene derivative}\) with \(N,N\)-diisopropyl dibenzylphosphoramidite and 1\(\text{-H-tetrazole}\) in dichloromethane followed by \textit{in situ} oxidation of the intermediate phosphite with \(m\)-chloroperbenzoic acid at \(-10\) °C to give the corresponding dibenzylphosphate derivative (m.p. 106-107°C) in 87% yield. Concomitant hydrogenolysis of the benzyl groups and hydrolysis of the isopropylidene acetal under \(H_2\) with 10% Pd/C in aqueous acetic acid followed by salification of the acidic product with diluted NaOH (pH meter control) provided the sodium salt of Xyl(2-phosphate)\(\beta\)1\(-\text{O-MN}\), [\(\alpha\)]\(_D\) \(-17^\circ\) (c1, water), as an amorphous solid in 71% overall yield.
Condensation of the major 2,3-O-isopropylidene derivative with 2,3,4,6-tetra-O-trichloroacetimidoyl-ß-D-galactopyranose (24) in toluene at 0°C under the catalysis of trimethylsilyl triflate gave the ß-linked disaccharide derivative in 73% yield. Mild hydrolysis of the isopropylidene acetal with aqueous acetic acid afforded the corresponding diol in 90% yield. This later compound was transesterified to give Galß1-4Xylß1-O-MN (m.p. 238-240°C) in 94% yield. Tin-mediated regioselective acetylation at O-3 of the Xyl residue of the disaccharide diol, as previously reported (25) for the synthesis of phosphorylated glycopeptides, followed by phosphorylation at O-2 as described above gave the corresponding fully protected disaccharide derivative in 60% overall yield. Subsequent hydrogenolysis followed by hydrazinolysis of the esters and salification with NaOH gave Galß1-4Xyl(2-phosphate)ß1-O-MN as its sodium salt, [αl]D -41° (c1, water) in 81% overall yield. All synthetic compounds have analytical data (NMR, mass, elemental analyses) fully consistent with the expected structures.

The following methoxyphenyl (MP)-digalactosides, analogs of the GAG-protein linkage region were prepared as previously described (26). The structure of these compounds is presented in Fig. 1C: Galß1-3Galß1-O-MP (5), Gal(4-sulfate)ß1-3Galß1-O-MP (6), Gal(6-sulfate)ß1-3Galß1-O-MP (7), Galß1-3Gal(4-sulfate)ß1-O-MP (8), Galß1-3Gal(6-sulfate)ß1-O-MP (9), Gal(6-sulfate)ß1-3Gal(6-sulfate)ß1-O-MP (10). The corresponding trisaccharides: GlcAß1-3Galß1-3Galß1-ß-O-MP (11) GlcAß1-3Gal(4-sulfate)ß1-3Galß1-ß-O-MP (12), GlcAß1-3Gal(6-sulfate)ß1-3Galß1-ß-O-MP (13), GlcAß1-3Galß1-3Gal(4-sulfate)ß1-O-MP (14), GlcAß1-3Galß1-3Gal(6-sulfate)ß1-O-MP (15), GlcAß1-3Gal(6-sulfate)ß1-3Gal(6-sulfate)ß1-O-MP (16) were synthesized as previously reported (27).

cDNA cloning and plasmid construction - The human GalT-I sequence was cloned by polymerase chain reaction (PCR) from a placenta cDNA library (Clontech, Palo Alto, CA) using a sense primer (5’-ATGTTCCTTCCGCGAGGAAAGCGGCAGGCAG-3’) together with an antisense primer (5’-TCAGCTGAATGTGCACCAGGGTGTGCGGTCTTG-3’) corresponding to the 5’ end and the 3’ end of the coding region of GalT-I described by Almeida et al. (5). The fragment amplified using Advantaq polymerase (Clontech) was subcloned into PCR2.1 (TA cloning kit (Invitrogen)) and sequenced on both strands. The cDNA sequence was 100% identical to that previously described (5). For the heterologous expression of the human GalT-I in P. pastoris, the full-length cDNA sequence was modified by PCR to include a SacII site and a Kozak consensus
sequence at the 5'-end and a XbaI site at the 3'-end, using appropriate oligonucleotides. The modified cDNA was then subcloned into the SacII-XbaI sites of the yeast expression vector pPICZB to produce pPICZ-GalT-I. Cloning of the full-length GlcAT-I cDNA and construction of the recombinant pPICZ-GlcAT-I were performed as previously described (19). Construction of amino acid substituted mutants of GlcAT-I was carried out using pPICZ-GlcAT-I as template with the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations. Mutagenic primers are presented in Table I. Mutants were systematically checked by sequencing and the various mutants were individually expressed in *P. pastoris* as described below. For the expression of the soluble catalytic domain of GlcAT-I (21), the sequence encoding T76 to V335 was amplified and subcloned into the SacII-XbaI sites of pPICZB. The active protein was expressed intracellularly in *P. pastoris* and purified from the cytosolic fraction as described below.

**Heterologous expression in the yeast *P. pastoris* and purification** - Each recombinant pPICZ vector was individually transformed into the *P. pastoris* SMD1168 yeast strain using the *P. pastoris* Easy Comp Transformation kit (Invitrogen). Transformants were selected on YPD-plates (1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose) containing 100 µg/ml of Zeocin (Invitrogen). The cells were grown in BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate (pH 6.0), 1.34 % (w/v) yeast nitrogen base and 1% (v/v) glycerol). Expression was induced by methanol (2%, v/v) for 48 h at 30°C in a rotary shaker (180 rpm). Yeast cells were broken with glass beads and further submitted to differential centrifugation as previously described (19). The 100,000 x g pellet corresponding to the membrane fraction was resuspended by Dounce homogenization in sucrose-HEPES buffer (0.25 M sucrose, 5 mM HEPES, pH 7.4). Protein concentration was evaluated by the method of Bradford (28) and membrane fractions were analyzed by SDS-PAGE, immunoblotting and used for kinetic analyses.

For purification of GlcAT-I, the supernatant from the 100,000 x g centrifugation containing the soluble GlcAT-I form (T76-V335) was dialyzed against buffer A (20 mM sodium phosphate, pH 7.0) at 4°C overnight and applied to a DEAE-Sephacel column (6 x 1.5 cm, Sigma), which had been equilibrated with the same buffer at a flow rate of 0.5 ml/min. The flow-through was applied onto a cation exchange support (Macro-Prep High-S, 11 x 21 cm, Bio-Rad) equilibrated with buffer A at a flow rate of 0.5 ml/min. After washing with buffer A and then with the same buffer containing 100
mM NaCl (5 column-volume), the protein was eluted with a linear gradient of 0.02 to 0.2 M sodium phosphate buffer pH 7.0 and analyzed by SDS-PAGE, Coomassie blue staining and immunoblotting. The protein was purified to apparent homogeneity and 0.05-0.1 µg protein/assay was used for kinetic analyses.

SDS-PAGE was performed under reducing conditions according to Laemmli (29). Immunoblot analysis was performed using a polyclonal anti-peptide antibody and alkaline phosphatase-conjugated anti-rabbit immunoglobulins as secondary antibodies, as previously described (19). The amount of recombinant wild-type and mutant GlcAT-I protein expressed in membrane fractions of yeast cells was evaluated from Western blot analysis using a calibration curve established with 0.01 to 0.10 µg protein purified protein run on the same gel.

Glycosyltransferase activity assays and kinetics – For GalT-I assays, 50-100 µg membrane proteins were incubated in 100 mM acetate buffer (pH 6.5) containing 5 mM MnCl₂, 1 mM UDP-Gal, 1 mM xyloside (compound I and 2) at 37°C for 60 min in a total volume of 100 µl. After incubation, the reaction was stopped by precipitating the proteins with 10 µl HCl 6 N. The samples were centrifuged for 10 min at 10,000 x g and an aliquot of the supernatant was analyzed by reverse-phase HPLC using a C₁₈ column (4.6 x 150 mm, 5 µm, Alltech Associates, Deerfield, IL) at a detection wavelength of 285 nm. The mobile phase was composed of 20 % (v/v) acetonitrile and 0.05 % (v/v) trifluoroacetic acid in water for the nonphosphorylated acceptor substrate (Xylβ1-O-MN) and 17 % (v/v) acetonitrile and 0.025 % (v/v) trifluoroacetic acid in water for the phosphorylated xyloside (Xyl(2-phosphate)β1-O-MN) at flow rate of 1 ml/min.

Activity of recombinant human GlcAT-I was evaluated using Galβ1-3Galβ1-4MP (compound 5) and each of the mono- (compound 6 to 9) or disulfated digalactosides (compound 10) as acceptor substrate. Incubations were performed in 100 mM acetate buffer with 50 mM MnCl₂, 50-100 µg membrane proteins (or 0.05-0.1 µg purified enzyme), 1 to 25 mM substrate and 1 mM UDP-GlcA in a total volume of 100 µl at 37°C for 60 min in a range of pH from 5.0 to 7.5. The mixtures were applied to a Sep-Pak C₁₈ cartridge (Waters, Milford, MA) before chromatographic analysis to remove UDP-GlcA. After the cartridge has been washed with 2 ml of water, the reaction product and the acceptor substrate were eluted by 1 ml of acetonitrile and further analyzed by gel filtration HPLC using a Superdex Peptide HR10/30 column (10 x 300 mm, Amersham Biosciences, Freiburg, Germany) with 0.02 M dipotassium phosphate buffer (pH 8.0) containing 0.2 M NaCl as eluent at
a flow rate of 0.3 ml/min. The reaction product was detected at a wavelength of 285 nm. We verified
that no loss of reaction product occurred upon Sep-Pak extraction.

For each set of experiments, control assays in which either the donor or the acceptor substrate
was omitted were systematically run under the same conditions. Addition of a GlcA or Gal residue
on the nonreducing end of acceptor substrates was verified by comparison of retention time with
that of the corresponding chemically synthesized standards (see Fig. 1B and 1C) and by the
associated radioactivity monitored by a LB 507A Berthold radioactivity detector, when incubations
were carried out in presence of UDP[14C]-GlcA or UDP[14C]-Gal (0.1 µCi) as donor substrate.
Quantitation of the reaction products was performed with calibration curves drawn with increasing
concentrations of the standards resolved under similar conditions. A time-course of the
glucuronosyltransferase and galactosyltransferase activity in membrane fractions showed that the
product formation was linear with time over 120 min. Initial rate data were subsequently taken after
a 60 min incubation time.

Apparent kinetic parameters \( (K_m, V_{max}) \) of GalT-I were determined using linear least-squares
regression analysis of double-reciprocal plots of initial velocity versus UDP-Gal (0 to 2 mM) at a
constant concentration of Xyl\( \beta \)1-O-MN (10 mM) and of initial velocity versus acceptor substrate
(0 to 25 mM) at a constant concentration of UDP-Gal (1 mM). Kinetic parameters \( (K_m, V_{max}) \) of
GlcAT-I were determined using linear least-squares regression analysis of double-reciprocal plots of
(1 mM) at optimum pH 6.5. Kinetic parameters of GlcAT-I towards Gal\( \beta \)1-3Gal(6-sulfate)\( \beta \)1-O-
MP were determined in a 0 to 2.5mM range of substrate at a constant concentration of UDP-GlcA
(1 mM) and optimum pH 5.5.

**Molecular Modeling** - The model structure of the GlcAT-I complex with the unsulfated and
sulfated digalactosides was built by energy minimization and molecular dynamics calculations based
on the initial structure of GlcAT-I (pdb code 1FGG (21)). The conformation with the lowest
estimated free energy of binding for each substrate was minimized using AMBER 7.0 program (30).
To avoid the missing residue regions in the pdb file, all minimizations were done keeping fixed all
residues outside a sphere of 12-Å radius from the sulfated molecule. Charges of Gal\( \beta \)1-3Gal(6-
sulfate) were calculated using GAUSSIAN94 package (31) and the HF/6-31 G* basis set. Atom-
centered charges were fitted with Antechamber of AMBER 7 software package (30). An automated
flexible docking of sulfated molecules into the active site of GlcAT-I was done using AutoDock 3.0 program using the genetic algorithm feature (32).
RESULTS

Xyl(2-phosphate)β1-O-MN is not a substrate for GalT-I - The activity of the recombinant human GalT-I expressed in *P. pastoris* was tested towards the unphosphorylated xyloside (Xyl1β-O-MN) by reverse-phase HPLC. A chromatogram of the HPLC resolution of the substrate and the reaction product (Galβ1-4Xyl1β1-O-MN) formed in the presence of UDP-Gal as cosubstrate is illustrated in Fig. 2A. The reaction product was identified by 1) comparison of retention time with that of the chemically synthesized standard, 2) associated radioactivity when UDP[14C]-Gal was used as donor substrate, 3) absence of detectable peak when substrate or cosubstrate was omitted from the assay. As expected, the results showed that the recombinant GalT-I was active towards Xyl1β-O-MN. Determination of the kinetic parameters of the recombinant enzyme led to apparent $V_{max}$ and $K_m$ values towards the acceptor substrate (Xylβ1-O-MN) of 47.2 pmol. min$^{-1}$. mg protein$^{-1}$ and 2.0 mM, respectively. Apparent $V_{max}$ and $K_m$ constants towards the donor substrate (UDP-Gal) were 46.3 pmol. min$^{-1}$. mg protein$^{-1}$ and 0.14 mM, respectively.

The phosphorylated derivative (Xyl(2-phosphate)β1-O-MN was next tested as acceptor substrate for GalT-I. Chromatographic analysis of the chemically synthesized standards (ie substrate and reaction product) showed that Xyl(2-phosphate)β1-O-MN and Galβ1-4Xyl(2-phosphate)β1-O-MN were resolved under our analytical conditions (Fig. 2B). The enzymatic assay indicated that, in contrast to the unphosphorylated xyloside, no activity could be observed when using the phosphorylated analog as a potential substrate for the recombinant enzyme. The same result was obtained in various conditions of assay: pH 5.0 to 7.5, substrate concentration range 0 to 50 mM, and Mn$^{2+}$ concentration 0 to 100 mM. This clearly indicates that GalT-I was unable to catalyze the transfer of Gal from UDP-Gal onto Xyl when this residue was phosphorylated on the C-2 position.

Galβ1-3Gal(6-sulfate)β1-O-MP is a preferred substrate for the human GlcAT-I - GlcAT-I catalyzes the transfer of GlcA provided by UDP-GlcA onto Gal2 of the GAG-protein linker region and was previously shown to recognize Galβ1-3Gal (Gal2-Gal1) disaccharide as minimum substrate (21). We analyzed the specificity of the human recombinant enzyme expressed in the yeast *P. pastoris* towards various sulfated analogs of Galβ1-3Galβ1-O-MP (Fig. 1B). A gel filtration HPLC assay was developed for the separation of the substrates and reaction products (Fig. 3). As expected, the recombinant GlcAT-I exhibited high activity towards the unsulfated Galβ1-3Galβ1-O-
MP substrate as shown in Table II. Interestingly, GlcAT-I efficiently catalyzed the transfer of GlcA onto the monosulfated derivative Galβ1-3Gal(6-sulfate)β1-O-MP (see HPLC chromatogram in Fig. 3 and Table II). By contrast, the recombinant enzyme exhibited no significant activity towards none of the other mono- or disulfated substrates tested, Gal(4-sulfate)β1-3Galβ1-O-MP, Gal(6-sulfate)β1-3Galβ1-O-MP, Galβ1-3Gal(4-sulfate)β1-O-MP, or the di-C-6 sulfated derivative. These data clearly indicate that the active site of the enzyme can accommodate a sulfate group only at the C-6 position of Gal1. The $V_{\text{max}}$ of the membrane-bound GlcAT-I for Galβ1-3Galβ1-O-MP and its C-6 sulfated analog were similar (345 and 303 nmol.min$^{-1}$.mg GlcAT-I protein$^{-1}$, respectively). By contrast, the $K_m$ of the membrane-bound GlcAT-I for Galβ1-3Gal(6-sulfate)β1-O-MP was 6-fold lower than that of its unsulfated derivative (Table II). Kinetic analyses performed on purified GlcAT-I confirmed that the enzyme exhibited a much higher affinity towards the sulfated derivative than towards the unsulfated compound (about 10-fold). Thus, our results show that GlcAT-I exhibited a markedly higher efficiency towards the sulfated substrate compared to its unsubstituted counterpart due to lower $K_m$ value suggesting that the 6-sulfated digalactoside was a better substrate than the unsulfated compound.

Identification of active site residues involved in recognition of sulfated and unsulfated derivatives - In an attempt to further delineate the structural determinants governing GlcAT-I specificity, we built a computer-aided model of the substrate-enzyme complex based on the X-ray structure of the catalytic domain of GlcAT-I bound to UDP (21). Figure 4 shows the superimposition of unsulfated and C-6-sulfated substrate in the acceptor binding site, pointing out residues in the vicinity of Gal1. This figure shows the dominant stacking interactions between the side-chain of W243 and Gal1 ring of the digalactoside acceptor substrate. Docking Galβ1-3Gal(6-sulfate) with GlcAT-I structure revealed that this compound adopted a position similar to that of the unsulfated substrate with possible stacking interaction with W243. Structural analysis of GlcAT-I had previously provided evidence for a dimeric organisation of this protein with residues E312-Q318 of one monomer extending into the active site of the other (21). In the related β1,3-glucuronosyltransferase GlcAT-P, the residues V320 and N321, which correspond to K317 and Q318 in GlcAT-I had also been suggested to contribute to acceptor substrate specificity (33). In GlcAT-I, Q318 of the second monomer was found in a position to form a hydrogen bond with the O-6 of Gal1. Molecular modeling suggested that this residue could also interact with the sulfate substituent of Galβ1-
3Gal(6-sulfate) (Fig. 4). In addition, computational modeling pointed out the presence of a conserved diglycine motif G222-G223 in the vicinity of O-4 and O-6 of Gal1 moiety although not at hydrogen bond distance. These residues form a cavity which can apparently accommodate the sulfated molecule. Based on these informations, we constructed a series of mutants to determine the importance of the different residues in the vicinity of Gal1 ring. Both conservative and non-conservative mutations were carried out and the consequences of these mutations were evaluated on GlcAT-I activity.

Upon expression in *P. pastoris*, immunoblot analysis showed that each mutant was expressed at similar level or even higher than the wild-type protein (Fig. 5A). Specific activities of wild-type GlcAT-I and mutants were normalized relative to the amount of expressed protein and was evaluated towards Galβ1-3Galβ1-O-MP and its analog sulfated on C-6 of the Gal1 unit (Fig. 5B). Replacement of W243 by alanine led to a complete loss of GlcAT-I activity towards both the unsulfated and sulfated digalactoside, indicating that this residue was critical for glucuronosyltransferase activity, in agreement with molecular modeling prediction. Substitution of this amino acid by a conservative phenylalanine residue slightly restored the enzyme activity towards the unsulfated and its C-6 sulfated analog to about 8 and 3 % of the initial activity, respectively (Fig. 5B). The importance of the conserved G222-G223 diglycine motif was analyzed by alanine replacement. The G222A mutant exhibited strongly reduced activity towards Galβ1-3Galβ1-O-MP and Galβ1-3Gal(6-sulfate)β1-O-MP (3.7- and 7.1-fold, respectively) (Fig. 5B). Substitution of G223 to alanine led to a near complete loss of GlcAT-I activity towards both substrates (Fig. 5B). The double G222-G223 to alanine mutation yielded a totally inactive enzyme, indicating that increasing the side chain of the residues at these positions prevents GlcAT-I activity (Fig. 5B). Finally, effects of the mutation of K317 and of the adjacent Q318 (belonging to the second GlcAT-I monomer) were also evaluated. Unexpectedly, the results indicated that alanine substitution of Q318 only slightly reduced enzyme activity towards the unsulfated analog and did not change that of Galβ1-3Gal(6-sulfate)β1-O-MP. By contrast, mutation of K317 to alanine led to a complete loss of GlcAT-I activity towards both substrates tested (Fig. 5B). Replacement of K317 by an arginine residue restored GlcAT-I activity to some extent (to about 15 % of the initial activity), emphasizing the importance of a positive charge at this position.
DISCUSSION

The biosynthesis of the common GAG-protein linkage region is a key step in the assembly of
PGs since completion of this tetrasaccharide sequence is essential for the conversion of core
proteins to functional PGs. Attempts to elucidate signal elements that regulate GAG initiation and
elongation have recently focused on the structure of the linkage region. In this study, we
investigated, for the first time, the influence of the two major modifications of the tetrasaccharide
sequence ie phosphorylation of Xyl and sulfation of Gal1/Gal2 on the activity of human GalT-I and
GlcAT-I enzymes, respectively. This could be achieved by the development of a stereo-controlled,
high-yielding synthesis of phosphorylated and sulfated analogs which have been tested as substrates
of the recombinant GalT-I and GlcAT-I expressed in yeast *P. pastoris*. This strategy has been
proved highly valuable for probing the specificity of other glycosyltransferases such as
mannosyltransferases (34).

A major finding of this study is that GalT-I did not show activity towards the C-2
phosphorylated xyloside, suggesting that the presence of this modification on the acceptor substrate
precludes recognition and/or transfer of Gal onto xyloside derivatives. This observation is consistent
with a biosynthetic mechanism in which phosphorylation would occur once Gal is attached to the
Xyl residue of the nascent oligosaccharide linkage. In agreement, studies of the biosynthesis of
human decorin expressed in rat fibroblasts by pulse-chase experiments, showed that
phosphorylation was prominent after the addition of the two Gal monosaccharides onto the Xyl
residue (35). On the other hand, these authors found that a proportion of the xylosylated decorin
intermediate was phosphorylated. However, this work did not indicate whether or not the
phosphorylated xylosylated decorin was further engaged in the GAG biosynthetic pathway. Since
we show that phosphorylated xyloside cannot serve as a substrate for GalT-I, it can be suggested
that phosphorylation may arrest the biosynthesis of some GAG chains and thus may represent a
regulatory mechanism in the biosynthesis rate of PGs. In a similar way, α-GalNAc capping of the
core tetrasaccharide primer (36) and 4,6-disulfatation of GalNAc residues of CS chains (37) have
been postulated to represent chain termination mechanisms. However, this issue awaits further
investigation.
In addition to phosphorylation, sulfation has been shown to occur on the Gal residues of the linkage tetrasaccharide region of numerous CS-PGs (see ref 9-15). Systematic synthesis of variously sulfated analogs of (Galβ1-3Gal)β1-O-MP offers a unique opportunity to examine whether sulfation is important with regard to the specificity of GlcAT-I. Interestingly, we demonstrate here that the efficiency of GlcAT-I was markedly enhanced when Gal1 of the acceptor substrate was sulfated on the C-6 position. The affinity of the full-length membrane-bound protein and purified enzyme was substantially higher for the sulfated acceptor substrate than for the nonsulfated analog, suggesting that the transfer of GlcA on the sulfated species would be favored. On the other hand, no GlcAT-I activity was observed when Gal1 was sulfated on C-4 position or when Gal2 was sulfated on either C-4 and/or C-6 position. The structure of GlcAT-I indicated that the majority of the hydrogen bond interactions between GlcAT-I and the acceptor substrate are through O-6, O-4 and O-3 hydroxyl group of Gal2, consistent with the idea that the presence of a sulfate substituent at any of these positions may prevent substrate binding. In agreement, no satisfactory docking of the sulfated digalactosides in GlcAT-I active site, except for Galβ1-3Gal(6-sulfate)β1-O-MP could be achieved. Since the catalytic efficiency of GlcAT-I was significantly higher with Galβ1-3Gal(6-sulfate) derivative than with the nonsulfated analog, we further investigated how the enzyme can accommodate this structure within the active site. For this purpose, systematic mutations of amino acid residues that are in the vicinity of Gal1, based on structural and molecular modeling, were carried out. Mutation of W243 residue highlighted the importance of the aromatic side-chain of tryptophan and that of phenylalanine in the W243F mutant. This residue appears essential in substrate positioning via stacking interaction with Gal1 in the case of both sulfated and unsulfated digalactoside. This result corroborates studies carried out on the related β1,3-glucuronosyltransferase GlcAT-P involved in the synthesis of HNK1 epitope emphasizing the importance of stacking interactions between F245 of GlcAT-P (corresponding to W243 in GlcAT-I) and the GlcNAc ring of the acceptor substrate, N-acetyllactosamine (Galβ1-4GlcNAc) (33). Accordingly, several studies indicate that aromatic residues are main determinants for specificity of glycosyltransferases such as cyclo-dextrin-glycosyltransferase (38). In addition, we showed that increasing the size of the side-chain of the two sequential glycine residues G222 and G223 (either individually or in combination) by alanine replacement strongly impaired GlcAT-I activity. Since these residues are not in hydrogen bond distance to the acceptor substrate, it can be suggested that
they play a role in the organization of the active site rather than being directly involved in interactions with the digalactoside acceptor substrate. In a model of GlcAT-P complexed to Lewis X, the fucose moiety of this saccharide structure clashes with G223 and G224 (corresponding to G222 and G223 in GlcAT-I), supporting the notion that these residues are important active site residues of the $\beta$1,3-glucuronosyltransferases (33).

Furthermore, structural analysis of GlcAT-I and GlcAT-P suggested that the C-terminus extending from the neighboring monomer may be involved in interaction with Gal1 or with GalNAc of the disaccharide acceptor substrate of these enzymes, respectively. In GlcAT-P, it has been shown that V320 and N321, which correspond to K317 and Q318 in GlcAT-I sequence play an important role in recognition of GlcNAc moiety. Unexpectedly, we showed that mutation of Q318, predicted to interact with $O$-6 of Gal1, into alanine had little effect on catalytic activity of GlcAT-I towards either sulfated or unsulfated digalactoside, indicating that the functional role of this residue is not critical for binding or specificity. By contrast, mutation of K317 produced deleterious effect on GlcAT-I activity emphasizing the contribution of this residue of the neighbour monomer in substrate recognition. Altogether, our results favors the idea that the stacking interactions between both unsulfated and $C$-6 sulfated digalactoside and W243 together with the presence of the sequential G222-G223 and K317 are essential for substrate recognition and GlcAT-I activity.

Our data suggest that sulfation is a key element influencing GlcAT-I activity, which may regulate the processing of PG biosynthesis. In a similar way, it has been demonstrated that sulfation is important with regard to the specificity of the glycosyltransferases involved in CS chains synthesis. Early studies from Kitagawa et al. (39) on a bovine serum $\alpha$-GalNAc-transferase using various CS oligosaccharides analogs (($\text{GlcA}$$\beta$1-$3\text{GalNAc}$$\beta$4-)$_n$) as substrates, showed that $C$-4 sulfation of the penultimate GalNAc unit markedly inhibited the enzyme activity. On the other hand, Sato et al. (40) showed strong activity of the recombinant CSGalNAcT-2 towards sulfated oligo- and polysaccharide CS substrates, suggesting that sulfation stimulates this CS-synthase and possibly elongation of CS chains. In a recent study, Seko et al. (41) demonstrated that the $\beta$1,4-galactosyltransferase 4 preferred keratan-sulfate related oligosaccharides to nonsulfated GlcNAc residues as acceptor substrates. Altogether, these and our data support the idea that sulfation represents a important regulatory mechanism of GAG processing and assembly.
In conclusion, we demonstrate, for the first time, that phosphorylation and sulfation critically determine the specificity of two glycosyltransferases involved in the biosynthesis of the GAG-protein-linkage region. This study emphasizes the potential role of selective modification of the tetrasaccharide primer sequence in the regulation of biosynthesis of the growing GAG linkage region and therefore in the conversion of nonglycanated to glycanated PGs. Elucidation of the structure and function of glycosyltransferases involved in the biosynthetic pathway of GAGs recently became a major challenge because of their implication in several pathologies and their potential as pharmacological targets. Glycosaminoglycan precursors are currently proposed as anti-amyloid or anti-thrombotic agents (42, 43) and in anti-cancer therapy (44). A better understanding of the specificity of the glycosyltransferases responsible for priming GAG biosynthesis achieved in this study thus represents an important step towards the development of GAG primers as potential therapeutic agents.

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**Footnotes**

1 Abbreviations

GAG, glycosaminoglycan; CS, chondroitin-sulfate; Gal, galactose; GalNAc, N-acetylgalactosamine; GalT-I, β1,4-galactosyltransferase-I; GlcA, glucuronic acid; GlcAT-I, β1,3-glucuronosyltransferase-I; GlcNAc, N-acetylglucosamine; HPLC, high performance liquid chromatography; HS, heparan-sulfate; O-MN, O-methoxynaphthyl; O-MP, O-methoxyphenyl; PG, proteoglycan; *P. pastoris, Pichia pastoris*; UDP-Gal, UDP-α-D-galactose; UDP-GlcA, UDP-α-D-glucuronic acid; Xyl, xylose.

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| Name          | Sense        | Antisense        |
|--------------|--------------|------------------|
| W243A        | 5' - CTTCCACACAGCA GCG GAGCCCAGCAGG - 3' | 5' - CCTGCTGGGCTCGCTGCTGTTGGAAG - 3' |
| W243F        | 5' - CTTCCACACAGCATTGCAGCCCAGCAGG - 3' | 5' - CCTGCTGGGCTCAGATGCTGTTGGAAG - 3' |
| G222A        | 5' - GTGGGGCTGGTG GCCGCTGCGATTGCAG - 3' | 5' - CTCGAATCGCAGCAGCCCACCAGCCCCAC - 3' |
| G223A        | 5' - GGGGCTGGTG GCCGCTGCGATTGCAG - 3' | 5' - CCTGCTGGGCTCAGATGCTGTTGGAAG - 3' |
| G222/G223A   | 5' - GTGGGGCTGGTG GCCGCTGCGATTGCAG - 3' | 5' - CCTGCTGGGCTCAGATGCTGTTGGAAG - 3' |
| K317A        | 5' - GAAGCCCAAGATGCAGGAGGAGCAGCTG - 3' | 5' - CTCGAATCGCAGCAGCCCACCAGCCCCAC - 3' |
| K317R        | 5' - GAAGCCCAAGATGCAGGAGGAGCAGCTG - 3' | 5' - CTCGAATCGCAGCAGCCCACCAGCCCCAC - 3' |
| Q318A        | 5' - GCCCAAGATGAAGGAGGGAGCAGCTG - 3' | 5' - CTCGAATCGCAGCAGCCCACCAGCCCCAC - 3' |
|             | 5' - CTCGAATCGCAGCAGCCCACCAGCCCCAC - 3' |
TABLE II

Kinetic analysis of GlcAT-I towards Galβ1-3Galβ1-O-MP and Galβ1-3Gal(6-sulfate)β1-O-MP

Membranes of recombinant yeast cells (0.25 μg GlcAT-I per assay) were incubated with increasing concentrations of Galβ1-3Galβ1-O-MP (0-25 mM) or Galβ1-3Gal(6-sulfate)β1-O-MP (0-5 mM) at a constant concentration of UDP-GlcA (1 mM). The product of the reaction was analyzed by gel filtration-HPLC and quantitated as described in “Experimental procedures”. The results are the mean of two independent assays in duplicate.

| Substrate | Galβ1-3Galβ1-O-MP | Galβ1-3Gal(6-sulfate)β1-O-MP |
|-----------|-------------------|-------------------------------|
|           | $K_m$ (mM) | $V_{max}$ (nmol.min$^{-1}$.mg$^{-1}$) | $V_{max}/K_m$ (μl.min$^{-1}$.mg$^{-1}$) | $K_m$ (mM) | $V_{max}$ (nmol.min$^{-1}$.mg$^{-1}$) | $V_{max}/K_m$ (μl.min$^{-1}$.mg$^{-1}$) |
|          |            |                                |                                |            |                                |                                |
|          | 2.10       | 345                            | 1.64                           | 0.36        | 303                            | 842                            |
LEGENDS OF FIGURES

FIG. 1. Schematic representation of the GAG-protein linkage region of PGs and chemical structures of the synthesized analogs. (A) Structure of the tetrasaccharide sequence attached to specific serine of consensus serine-glycine residues of the core protein. Potential sulfation and phosphorylation sites are marked by arrows. The glycosyltransferases involved are indicated and the enzymes used in this study are underlined. (B) Chemical structure of the unphosphorylated and phosphorylated compounds tested as substrates of GalT-I (numbers 1 and 2) and of the corresponding reaction products (numbers 3 and 4). (C) Chemical structure of the unsulfated and sulfated compounds used as substrates of GlcAT-I (numbers 5 to 10) and of the corresponding reaction products (numbers 11 to 16).

FIG. 2. HPLC chromatogram for activity measurement of recombinant human GalT-I towards phosphorylated and unphosphorylated xyloside. Membranes of P. pastoris yeast cells expressing the recombinant enzyme were incubated in the presence of acceptor substrate and of UDP-Gal as described under "Experimental Procedures". (A) Chromatograms of the HPLC resolution of the reaction product after incubation of GalT-I with UDP-Gal (peak 1) and Xylβ1-O-MN (peak 3). Peak 2 corresponds to the reaction product or to the synthetic standard Galβ1-4Xylβ1-O-MN. Control assay corresponds to an incubation in which UDP-Gal was omitted. (B) Chromatograms of the HPLC resolution of the reaction product after incubation of GalT-I with UDP-Gal (peak 1) and Xyl(2-phospate)β1-O-MN (peak 5). Peak 4 corresponds to the synthetic standard Galβ1-4Xyl(2-phosphate)β1-O-MN. Control assay corresponds to an incubation in which UDP-Gal was omitted.

FIG. 3. HPLC chromatogram for activity measurement of recombinant human GlcAT-I towards the sulfated digalactoside Galβ1-3Gal(6-sulfate)β1-O-MP. Membranes of P. pastoris yeast cells expressing the recombinant enzyme (0.25 µg of GlcAT-I) were incubated in the presence of acceptor substrate and of UDP-GlcA as described under "Experimental Procedures". Chromatograms of the HPLC resolution of the reaction product after incubation of GlcAT-I with UDP-GlcA and Galβ1-3Gal(6-sulfate)β1-O-MP (peak 2) are shown. Peak 1 corresponds to the
synthetic standard GlcAβ1-Galβ1-3Gal(6-sulfate)β1-O-MP or to the reaction product of the enzymatic reaction. Control assay corresponds to an incubation in which acceptor substrate was omitted.

FIG. 4. **Molecular modeling of the acceptor substrate binding site of GlcAT-I.** Superposition of the Galβ1-3Gal (green) and Galβ1-3Gal(6-sulfate) (blue) in the GlcAT-I active site. Residues G222, G223 and W243 belong to the first monomer of the GlcAT-I structure, residues K317 and Q318 to the second. The figure was built with DeepView (45) (web site http://www.expasy).

FIG. 5. **Expression and activity of wild-type GlcAT-I and mutants.** (A) SDS-PAGE and immunoblot analysis of membrane fraction of recombinant yeast cells expressing wild-type (WT), and GlcAT-I mutants probed with anti-GlcAT-I antibodies. 2.0 µg membrane protein were loaded per lane. (B) Activity of wild-type and mutant. enzyme activity was evaluated in the presence of Galβ1,3Galβ1-O-MP (5 mM, grey bars) or Galβ1,3Gal(6-sulfate)β1-O-MP (1 mM, dark bars) and UDP-GlcA (1 mM) as described under "Experimental procedures". Values are expressed as nmol.min⁻¹.mg⁻¹ GlcAT-I protein. GlcAT-I was quantitated using a calibration curve established with purified protein immunorevealed in the same conditions. Results are the mean ± SD of three assays and are representative experiments performed on two different batches of protein. ND, not detectable.
Figure 1

A

\[
\text{GlcAT-I} \quad \text{GalT-II} \quad \text{GalT-I} \quad \text{XylT-I}
\]

\[
\text{Gal1Gal2} \quad \text{Gal1Gal2GlcA} \quad \text{Xyl} \quad \text{Gal1} \quad \text{Gal2} \quad \text{Gal1}
\]

B

1. \(R = H\)
2. \(R = \text{PO}_3\text{Na}_2\)
3. \(R = H\)
4. \(R = \text{PO}_3\text{Na}_2\)

C

5. \(R = R^1 = R^2 = R^3 = H\)
6. \(R = R^1 = R^2 = H, R^3 = \text{SO}_3\text{Na}\)
7. \(R = R^1 = R^3 = H, R^2 = \text{SO}_3\text{Na}\)
8. \(R = R^2 = R^3 = H, R^1 = \text{SO}_3\text{Na}\)
9. \(R = \text{SO}_3\text{Na}, R^1 = R^2 = R^3 = H\)
10. \(R = \text{SO}_3\text{Na}, R^1 = R^3 = H\)
11. \(R = R^1 = R^2 = R^3 = H\)
12. \(R = R^1 = R^2 = H, R^3 = \text{SO}_3\text{Na}\)
13. \(R = R^1 = R^3 = H, R^2 = \text{SO}_3\text{Na}\)
14. \(R = R^2 = R^3 = H, R^1 = \text{SO}_3\text{Na}\)
15. \(R = \text{SO}_3\text{Na}, R^1 = R^2 = R^3 = H\)
16. \(R = R^2 = \text{SO}_3\text{Na}, R^1 = R^3 = H\)
Figure 2

A

B

A, B 285 nm

Standard
Assay
Control assay

Assay

Standard
Control assay

1 2 3 4 5

time (min)

0 0.1 0.2 0.3

A 285 nm
Figure 5

A

B

kDa

WT  W243A  W243F  G222A  G223A  G222-223A  K317A  K317R  Q318A

mmol.min⁻¹.mg prot⁻¹

WT  W243A  W243F  G222A  G223A  G222-223A  K317A  K317R  Q318A
Phosphorylation and sulfation of oligosaccharide substrates critically influence the activity of human β1,4-galactosyltransferase 7 (GalT-I) and β1,3-glucuronosyltransferase I (GlcAT-I) involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans

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