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Core Glycosylation of Collagen Is Initiated by Two β(1-*O*)Galactosyltransferases▽†

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Collagen is a trimer of three left-handed alpha chains representing repeats of the motif Gly-X-Y, where (hydroxy)proline and (hydroxy)lysine residues are often found at positions X and Y. Selected hydroxylysines are further modified by the addition of galactose and glucose-galactose units. Collagen glycosylation takes place in the endoplasmic reticulum before triple-helix formation and is mediated by β(1-*O*)galactosyl- and α(1-2)glucosyltransferase enzymes. We have identified two collagen galactosyltransferases using affinity chromatography and tandem mass spectrometry protein sequencing. The two collagen β(1-*O*)galactosyltransferases corresponded to the GLT25D1 and GLT25D2 proteins. Recombinant GLT25D1 and GLT25D2 enzymes showed a strong galactosyltransferase activity toward various types of collagen and toward the serum mannose-binding lectin MBL, which contains a collagen domain. Amino acid analysis of the products of GLT25D1 and GLT25D2 reactions confirmed the transfer of galactose to hydroxylysine residues. The GLT25D1 gene is constitutively expressed in human tissues, whereas the GLT25D2 gene is expressed only at low levels in the nervous system. The GLT25D1 and GLT25D2 enzymes are similar to CEECAM1, to which we could not attribute any collagen galactosyltransferase activity. The GLT25D1 and GLT25D2 genes now allow addressing of the biological significance of collagen glycosylation and the importance of this posttranslational modification in the etiology of connective tissue disorders.

Collagens are the most abundant proteins in the human body. To date, 29 types of collagen have been described, which are encoded by at least 44 genes (21, 37, 45). Collagens are characterized by domains representing repeats of the triplet Gly-X-Y, where proline and lysine are often found at positions X and Y. The Gly-X-Y repeats are not confined to collagens but are also found in several other proteins, such as the hormone adiponectin (29), the mannose-binding lectin (MBL) (11), the C1q complement protein (35), the COLQ subunit of the acetylcholine esterase complex (4), and the surfactant proteins SP-A and SP-D (11).

After synthesis in the endoplasmic reticulum (ER), three procollagen subunits associate to build a right-handed triple helix. However, before the formation of the triple-helix structure, the nascent procollagen polypeptides undergo several posttranslational modifications. These modifications comprise the hydroxylation of selected proline (20) and lysine (33) residues, which are catalyzed by three prolyl-4-hydroxylases (17), one prolyl-3-hydroxylase (46), and three lysyl hydroxylases (43). Hydroxylysine can be further modified by the addition of the monosaccharide Gal(β1-*O*) or the disaccharide Glc(α1-2)Gal(β1-*O*) (39).

Whereas the glycosylation of collagen was first described by Grassmann and Schleich in 1935 (9) and the structure of the glycan determined by Spiro in 1967 as being Glc(α1-2)Gal(β1-*O*)Hyl (40), the molecular nature of the collagen glycosyltransferase enzymes has remained elusive up to now. Collagen galactosyltransferase (ColGalT) and glucosyltransferase activities have been characterized using partially purified proteins (24, 31, 32), which appeared to be unstable. Recently the lysyl hydroxylase 3 (LH3) enzyme has been shown to catalyze a modest galactosyl and glucosyltransferase activity, suggesting that this enzyme is a combined hydroxylase and glycosyltransferase (12).

Prolyl and lysyl hydroxylation contribute to the stability of the collagen triple helix, where hydroxylysine is essential for the cross-linking of collagen molecules, thus ensuring the strength of collagen fibrils (28). In contrast, the biological significance of collagen glycosylation is still unclear. The collagen domain of adiponectin and mannose-binding lectin also carry glycosylated hydroxylysine residues, which appear to be important for the oligomerization and proper secretion of these proteins (6, 29).

The importance of collagen posttranslational modifications is reflected by the diseases caused by defective collagen modifying enzymes. Mutations of the *LH1* lysyl hydroxylase 1 gene lead to the connective tissue disorder Ehlers-Danlos syndrome type VI (14), and mutations in the *LH2* lysyl hydroxylase 2 gene lead to the Bruck syndrome (44). A deficiency in the prolyl 3-hydroxylase 1 gene causes a severe form of osteogenesis imperfecta (5). The availability of the collagen glycosyltransferase genes will enable comprehensive investigation of this posttranslational modification in cellular and animal models and possibly in human diseases.

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MATERIALS AND METHODS

Affinity chromatography. Collagen glycosyltransferases were enriched by affinity chromatography as described by Myllyla et al. (1, 22, 24). Briefly, 10-day-old chicken embryos were homogenized in 225 mM mannitol, 75 mM sucrose, 50

μM dithiothreitol (DTT), and 50 mM Tris-HCl, pH 7.4, at 4°C and centrifuged at 15,000 × *g* for 40 min. Supernatants were filtered and proteins precipitated in 60% $(NH_4)_2SO_4$. The pellets obtained after 20 min of centrifugation at 15,000 × *g* were dissolved in 0.2 M NaCl, 50 μM DTT, 1% glycerol, 20 mM Tris-HCl, pH 7.4, and dialyzed overnight against 2.5 liters of enzyme buffer (0.15 M NaCl, 10 mM $MnCl_2$, 50 M DTT, 1% glycerol, 50 mM Tris-HCl, pH 7.4). The chicken protein extracts were loaded on a column of agarose-bound bovine Achilles collagen type I fragments as described previously (32). The column was washed with 5 volumes of enzyme buffer containing 500 μM UDP, followed by elution with 0.1% acetic acid. Collected fractions were immediately neutralized with 1 M Tris (pH 8.0).

MS peptide analysis. The eluted fractions from the affinity chromatography were desalted and concentrated with Amicon Ultra 10 cartridges (Millipore). Two-microgram portions of protein were reduced in 0.6 M Tris (pH 8.5)–50 mM DTT for 5 min at 80°C and alkylated for 40 min at room temperature in the dark by the addition of iodoacetamide (final concentration 200 mM; Sigma-Aldrich) and desalted by adding 9 volumes of ice-cold methanol for 18 h on ice. Alkylated proteins were digested for 18 h at 37°C with 0.01 μg trypsin (Roche). ZipTip (Millipore) purified peptides were then analyzed by liquid chromatography-mass spectrometry (MS). The desalted peptide digest was adjusted to 0.2% formic acid–3% acetonitrile (ACN) and directly injected onto a custom packed 80-mm by 0.075-mm ProntoSil-Pur C18 AQ (3 μm, 200 Å) column (Bischoff GmbH, Leonberg, Germany), connected to an LTQ-ICR-FT mass spectrometer (Thermo Scientific, Bremen, Germany). The peptides were eluted with a binary gradient of solvents A (3% ACN, 0.2% formic acid) and B (80% ACN, 0.2% formic acid) using an Eksigent-Nano high-performance liquid chromatography (HPLC) system (Eksigent technologies, Dublin, Ireland). The column was flushed for 16 min at a flow rate of 500 nl/min with 100% buffer A. Buffer B was increased to 3% over 5 min, to 60% over 50 min, and to 100% over 3 min and then held at 100% for 7 min. During gradient elution, the flow rate was maintained at 200 nl/min. The mass spectral data were acquired in the mass range of 300 to 2,000 *m/z*. Datum-dependent MS/MS spectra for up to four of the most intense ions with a higher charge state than 1+ were recorded using collision-induced dissociation. Target ions already selected for MS/MS were dynamically excluded for 60 s. Peptide signals exceeding 500 counts were subjected to collision-induced dissociation with a normalized collision energy of 32%. MS and MS/MS data were searched using Mascot Server 2.1 (Matrix Science, London, United Kingdom) as the search engine. Modifications used include carbamidomethylation (Cys, fixed) and oxidation (Met, variable). The monoisotopic masses of +2 and +3 charged peptides were searched with a peptide tolerance of 2 ppm and an MS/MS tolerance of 0.8 Da. MS/MS spectra were searched against the UniRef100 20051018 database (2,764,545 sequences; 1,015,909,965 residues) downloaded from the European Bioinformatics Institute (http://www.ebi.ac.uk/uniprot/database/download.html) and the *Gallus gallus* predicted protein database (ftp://ftp.ensembl.org/pub/release-51/fasta/gallus_gallus/pep/).

Cloning and protein expression. The *GLT25D2*, *LH3*, and *MBL* cDNAs were purchased from the RZPD repository (Berlin, Germany). The *GLT25D1* and *cerebral endothelial cell adhesion molecule 1 (CEECAM1*) cDNAs were cloned by reverse transcription-PCR (RT-PCR) from human fibroblast total RNA using the primers 5′-ATCTGAATTCCCTTTAAGGCGCGGCCAGAGTC-3′ and 5′-ATGTCTAGATGGAGCCTGGGCCACCGATG-3′ for *GLT25D1* and 5′-CGTAGAATTCGAGAGCTCCGGGGGCCGCT3′ and 5′-GACTATCTAGAGTAGTGGCCTGCTCCTGGAC-3′ (Microsynth, Switzerland) for *CEECAM1*. The RT-PCR products were subcloned as EcoRI-XbaI fragments into the pFastBacI baculovirus transfer vector (Invitrogen). The *MBL* cDNA was subcloned into the EcoRI site of the pFmel-protA vector (48) to yield a protein A fusion protein. The corresponding 732-bp *MBL* fragment was amplified with the primers 5′-ATCGAATTCATGGTGGCAGCGTCTTACTC-3′ and 5′-ATCGAATTCAGGAGGGCCTGAGTGATATG-3′. Recombinant baculoviruses were produced in *Spodoptera frugiperda* Sf9 cells as described previously (13). Protein A-tagged MBL was coexpressed together with LH3, purified from the supernatant of infected Sf9 cells by immunoglobulin G Sepharose chromatography (48), and subsequently used as an acceptor for the enzymatic activity assay. The expression of the recombinantly expressed enzymes was analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Prior to electrophoresis, proteins were enriched by concanavalin A Sepharose (GE Healthcare) chromatography. Protein bands were excised from the SDS-PAGE gel, digested in gel with trypsin according to the method of Shevchenko et al. (34), and identified by MS peptide analysis.

Preparation of ColGalT acceptors. Bovine Achilles collagen type I, bovine nasal septum collagen type II, and human placenta collagen types III, IV, and V (Sigma) were deglycosylated by trifluoromethane sulfonic acid (TFMS)-mediated cleavage (7, 38). Acceptor proteins (50 μg) were lyophilized, followed by an incubation in a dry ice-ethanol bath for 20 min. Proteins were dissolved in 50 μl TFMS-toluene (16.6:1 [vol:vol]) (Sigma-Aldrich). Reactions were subsequently incubated at −20°C for 24 h and then neutralized with 150 μl pyridine-H_2O (2:1 [vol:vol]) in the dry ice-ethanol bath, followed by 15 min of incubation on ice.

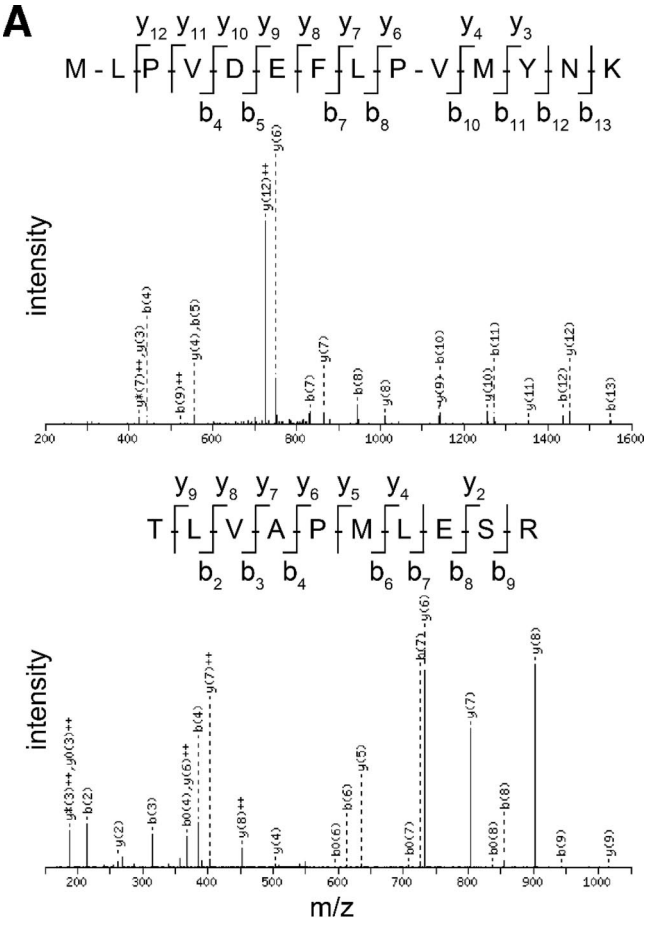

FIG. 1. ColGalT identification by mass spectrometry. Proteins isolated by affinity chromatography were analyzed by liquid chromatography-MS. (A) Peptide fragment spectra of two peptides identifying GLT25D2. (B) Protein sequence of *Gallus gallus* GLT25D2. The two identifying peptides are shaded in gray, the four potential N-glycosylation sites are underlined, and the ER retrieval signal is shown in bold.

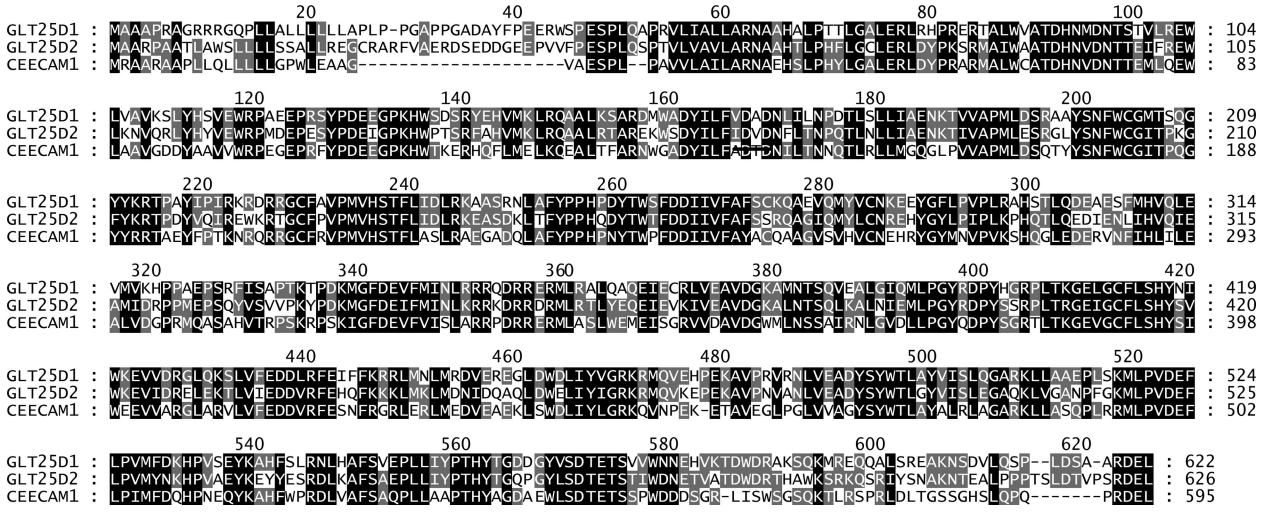

FIG. 2. Protein alignment. The three putative human ColGalT enzymes share a high degree of sequence identity (63% between GLT25D1 and GLT25D2, 50% between GLT25D2 and CEECAM1, and 55% between GLT25D1 and CEECAM1). The proteins include the C-terminal RDEL ER retrieval motif. Black squares represent amino acids identical or similar in all three proteins; gray squares represent amino acids identical or similar in two of the proteins.

The sample was mixed with 400 μl 50 mM ammonium acetate and dialyzed overnight against 2.5 liters of 50 mM ammonium acetate.

Collagen glycosyltransferase assays. Baculovirus-infected Sf9 cells were lysed in 1% Triton X-100–TBS (pH 7.4) for 10 min on ice, and the postnuclear supernatant was used as an enzyme source. Collagen was heat denatured for 10 min at 60°C in sodium acetate (pH 6.8) and rapidly cooled to 0°C before use. Assays were performed with 10 μl of Sf9 postnuclear supernatant in a final volume of 100 μl containing 0.5 mg/ml collagen acceptors, 60 μM UDP-Gal or UDP-Glc, 50,000 cpm UDP-[^{14}C]Gal or UDP-[^{14}C]Glc (GE Healthcare), 10 mM $MnCl_2$, 20 mM NaCl, 50 mM morpholinepropanesulfonic acid (pH 7.4), and 1 mM DTT. Reactions were incubated for 3 h at 37°C and stopped by the addition of 500 μl of ice-cold 5% TCA–5% phosphotungstic acid. Enzymatic activity assays for K_m analysis were performed as described above but with various amounts of either collagen type I or UDP-Gal as substrates.

Amino acid analysis. The reaction products of the collagen galactosyltransferase assays were hydrolyzed in 4 M NaOH for 72 h at 105°C, and the resulting single amino acids were derivatized with 9-fluorenylmethoxy carbonyl according to the method of Bank et al. (2). Reverse-phase HPLC (LaChrom Hitachi; Merck) of single amino acids was performed on an ODS Hypersil column (150 by 3 mm, 3-μm particle size; Thermo Electron Corporation) at 40°C. The galactosylated Hyl (GHyl) and galactosyl-glucosylated Hyl (GGHyl) standards were kindly provided by Ruggero Tenni (University of Pavia) (42). Amino acids were separated at a flow rate of 0.2 ml/min using a gradient elution with the solvents 0.5 M citric acid, 5 mM $(CH_3)_4NCl$, pH 2.85 (A); 80% of 20 mM sodium acetate trihydrate, 5 mM $(CH_3)_4NCl$, pH 4.5, 20% of methanol (B); and 100% of ACN (C). Radiolabeled [^{3}H]Val and [^{14}C]Tyr (Moravek Biochemicals and Radiochemicals) were used as internal standard. Radioactivity was counted in a β counter (Tri-Carb 2900TR; Packard). For β-galactosidase digestion of GHyl, hydrolyzed amino acids were loaded on AG 50W-X8(H^+) resin (Bio-Rad), washed with 0.8% acetic acid, and eluted with 5% ammonia. After removal of ammonia by lyophilization, the samples were digested with 10 mU of bovine testis β-galactosidase (QA-Bio, San Mateo, CA) in 100 mM sodium citrate, pH 4.3, for 16 h at 37°C. Liberated Gal was separated from GHyl by passage through AG 50W-X8(H^+), whereas GHyl was released by elution with 5% ammonia.

RNA interference. Lentivirus particles expressing the short hairpin RNA constructs TRCN0000034884, TRCN0000034885, TRCN0000034887, and TRCN0000034888, targeting human *GLT25D1* (MISSION shRNA NM_024656; Sigma) were produced in HEK293T cells as described previously (10). Aliquots of 500 μl of lentivirus-producing HEK293T cell supernatants were added to 60,000 HeLa cells for 24 h. Cells expressing the short hairpin RNA constructs were selected by treatment with 2.5 μg/ml of puromycin for 10 days. Silencing of the *GLT25D1* gene was monitored by quantitative RT-PCR (SYBR Green JumpStart *Taq* ReadyMix; Sigma) using the primers 5′-ATTGCGCGCCCACAGCAC-3′ and 5′-GGTGGGAGCCGAGATGAAGC-3′. The expression of the GLT25D2 and glyceraldehyde-3-phosphate dehydrogenase genes in HeLa cells was determined using the primers 5′-GATAACATTGACCAGGCTCAG-3′, 5′-

TABLE 1. ColGalT activities measured in Sf9 cell lysates[a]

Collagen used	TFMS[b]	ColGalT activity (pmol · min^{-1} · mg protein^{-1})			
		Sf9[c]	GLT25D1	GLT25D2	CEECAM1
None (no acceptor)		1.5 ± 0.6	1.9 ± 0.7	0.9 ± 0.4	0.5 ± 0.1
Type I	−	10.5 ± 1.8	43.7 ± 12.0	57.2 ± 8.5	8.7 ± 2.0
	+	5.0 ± 3.0	61.4 ± 18.2	18.0 ± 5.4	2.7 ± 1.9
Type II	−	6.5 ± 0.8	36.6 ± 3.7	35.1 ± 3.8	3.3 ± 1.3
	+	3.4 ± 0.8	67.0 ± 16.5	22.1 ± 8.8	2.8 ± 0.8
Type III	−	2.9 ± 0.9	31.7 ± 5.8	25.2 ± 6.8	1.8 ± 0.8
	+	11.0 ± 3.6	244.7 ± 25.7	102.1 ± 17.6	6.5 ± 1.7
Type IV	−	1.7 ± 1.4	3.6 ± 2.2	3.6 ± 0.9	1.9 ± 0.7
	+	12.9 ± 4.6	433.2 ± 49.6	164.2 ± 43.3	8.4 ± 2.4
Type V	−	2.3 ± 0.7	2.7 ± 0.3	2.3 ± 0.4	0.7 ± 0.1
	+	2.7 ± 1.6	18.6 ± 3.6	4.7 ± 1.5	2.0 ± 1.1

[a] Each value represents the average ± standard deviation for four assays.
[b] TFMS-mediated deglycosylation.
[c] Sf9 cells infected with an empty baculovirus were used.

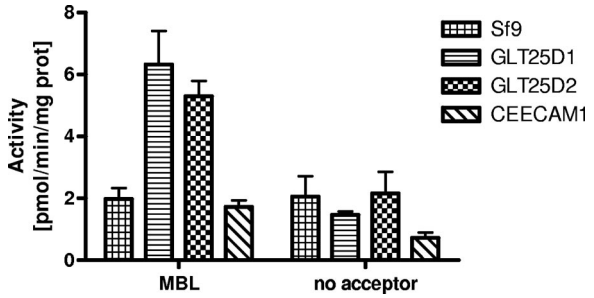

FIG. 3. ColGalT activity toward MBL. MBL was produced in Sf9 cells coinfected with a baculovirus expressing LH3. A ColGalT activity assay was performed as described in Materials and Methods. Bars indicate the means for four assays. Error bars indicate the standard deviations.

CCCAAAAGGATTGGCTCCAAC-3′, 5′-ATGCTGGCGCTGAGTACGTCG TG-3′, and 5′-GTGATGGCATGGACTGTGGTCAT-3′, respectively.

Northern blotting. The *GLT25D1*, *GLT25D2*, and *CEECAM1* cDNA probes were synthesized by PCR using the primer pairs 5′-GATGAGGCCGAGAGCT TCATGC-3′ and 5′-GCATGAAGCTCTCGGCCTCATC-3′, 5′-AAGCAGGC ATCCAGATGTACC-3′ and 5′-TCCAGCTGAGCCTGGTCAATG-3′, and 5′-GTGGATGGCTGGATGCTCAAC-3′ and 5′-GACTATCTAGAGTAGTGGC CTGCTCCTGGAC-3′, respectively. The resulting 676-bp-long *GLT25D1*, 559-bp-long *GLT25D2*, and 785-bp-long *CEECAM1* probes were labeled with [α-^{32}P]dCTP (Hartman Analytic, Germany) by random priming (Stratagene). Multiple human tissue RNA blots (MTE array 3 [BD Bioscience] and First Choice Northern Human Blot 1 [Ambion]) were prehybridized with the QuikHyb hybridization solution (Stratagene) containing 100 μg/ml ultra-pure herring sperm DNA (Invitrogen) for 1 h at 65°C and then hybridized with 5×10^5 cpm of each labeled probe overnight at 65°C. The arrays were washed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS up to 60°C and exposed on BioMax XAR film (Kodak) for 24 h at −80°C.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper correspond to the GenBank/EBI data bank entries with the accession numbers NM_024656, NM_015101, and NM_016174.

RESULTS

ColGalT identification. Most glycosylation pathways have been characterized at the molecular level over the past decades. However, the genes encoding the glycosyltransferases involved in the glycosylation of Hyl in collagen have remained unknown up to now. Here we have applied a cloning strategy based on the enrichment of proteins by affinity chromatography, peptide sequencing, and heterologous expression of isolated candidate proteins. The enrichment procedure followed the method of Risteli et al. (32) using immobilized denatured collagen type I to capture the ColGalT. Homogenates of 10-day-old chicken embryos were used as a source of ColGalT enzyme, as applied previously (1, 24). To identify potential ColGalT enzymes among the proteins enriched by affinity chromatography, we selected proteins sharing sequence homology with known glycosyltransferases. We also focused on proteins containing ER localization motifs, considering the cellular localization of collagen glycosylation, and on proteins containing N-glycosylation sites, since it was shown that the ColGalT activity could by enriched by concanavalin A lectin chromatography (30).

One of the candidate proteins identified by tandem MS peptide sequencing was the putative glycosyltransferase GLT25D2 (Fig. 1A) (see Fig. S1 in the supplemental material). GLT25D2 is a type II transmembrane protein of 626 amino

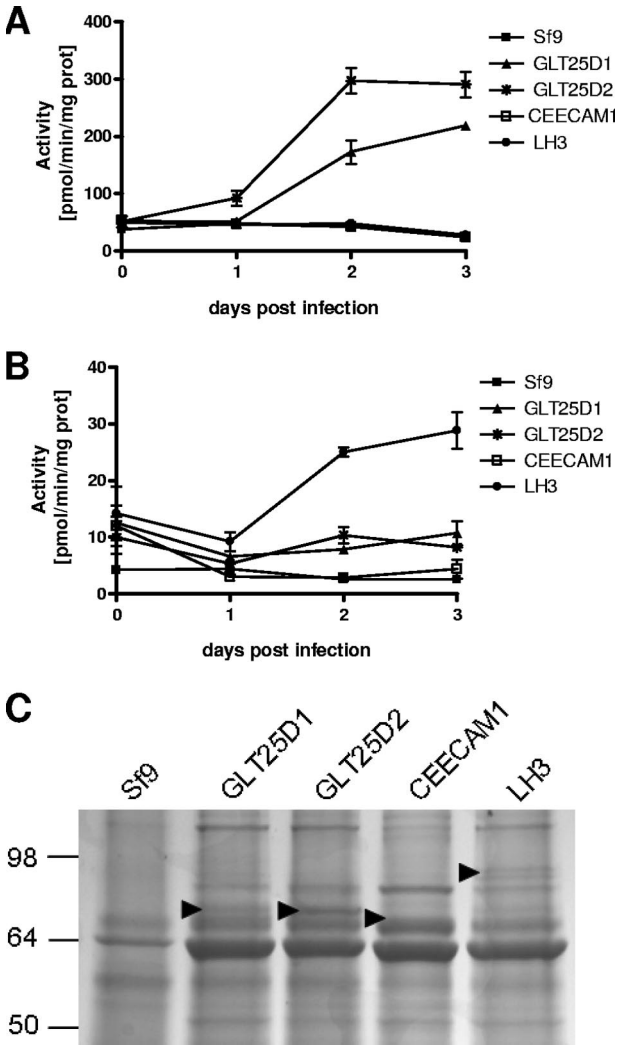

FIG. 4. Time course of baculovirus-mediated protein expression in Sf9 cells. ColGalT activity (A) or collagen glucosyltransferase activity (B) was measured in cells expressing GLT25D1, GLT25D2, CEECAM1, or LH3. Bovine Achilles collagen type I was used as an acceptor substrate. The activity measured in Sf9 cells infected with an empty baculovirus is shown in both panels with filled squares. Values indicate the means for four assays. Error bars indicate the standard deviations. (C) SDS-PAGE of recombinantly expressed proteins. Arrows indicate the recombinant protein bands, as confirmed by liquid chromatography-MS-mediated protein sequencing.

acids, including four N-glycosylation sites and the ER retention signal RDEL at the C terminus (Fig. 1B). No enzymatic activity was attributed to GLT25D2, but database annotations pointed to sequence homology with bacterial enzymes involved in lipopolysaccharide biosynthesis. Proteins similar to chicken GLT25D2 could be deduced from all metazoan genomes. In the human genome, GLT25D2 was found to be strongly similar to two proteins, namely, GLT25D1 and CEECAM1. The three proteins contained N-glycosylation sites and the ER retrieval signal RDEL at the C terminus and shared more than 50% sequence identity (Fig. 2).

ColGalT activity. The putative ColGalT activities of GLT25D1, GLT25D2, and CEECAM1 were assayed by expressing the

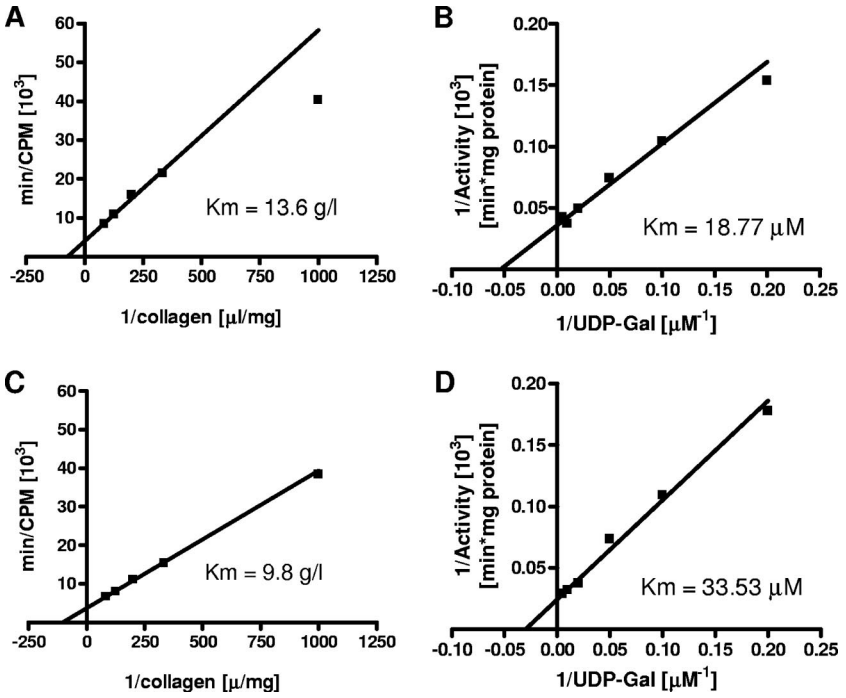

FIG. 5. Determination of the apparent K_m values of GLT25D1 and GLT25D2. (A) Lineweaver-Burk blot for GLT25D1 on collagen, with the calculated Michaelis-Menten constant of 13.6 g/liter. (B) Lineweaver-Burk blot for GLT25D1 on UDP-Gal, with the calculated Michaelis-Menten constant of 18.77 μM. (C) Lineweaver-Burk blot for GLT25D2 on collagen type I, with the calculated Michaelis-Menten constant of 9.8 g/liter. (D) Lineweaver-Burk blot for GLT25D2 on UDP-Gal, with the calculated Michaelis-Menten constant of 33.53 μM.

three proteins as recombinant baculovirus in Sf9 insect cells. Five types of collagen were tested as possible acceptor substrates. Because native collagen is readily glycosylated to varying extents, we also included deglycosylated collagen preparations in the assays. GLT25D1 and GLT25D2 showed a strong ColGalT activity on all deglycosylated collagen acceptors tested, whereas CEECAM1 did not show any activity (Table 1). As expected, the ColGalT activities of GLT25D1 and GLT25D2 were lower when native collagen acceptors were used. Noteworthily, collagen type IV and collagen type V in the native form were hardly galactosylated by GLT25D1 and GLT25D2, suggesting that most Hyl residues were already glycosylated. In addition to true collagens, GLT25D1 and GLT25D2 but not CEECAM1 were able to transfer Gal to the serum protein MBL (Fig. 3), which contains four Hyl sites in its collagen domain, showing that ColGalT activity was not limited to large collagen acceptors. The nucleotide sugars UDP-GlcNAc and UDP-GalNAc were also tested as possible donor substrates. but no GlcNAc or GalNAc transfer to collagen could be detected (data not shown).

We also tested the ColGalT activity of the human LH3 enzyme, which had been reported to catalyze three reactions on collagen, namely, the hydroxylation of Lys plus the β(1-O)galactosylation and α(1-2)glucosylation of Hyl (12, 47). Surprisingly, we could not detect any significant ColGalT activity for LH3 under our assay conditions using bovine Achilles collagen type I as an acceptor (Fig. 4A). However, as described previously (12, 47), we did measure a low collagen glucosyltransferase activity for LH3, whereas GLT25D1, GLT25D2, and CEECAM1 failed to show any significant collagen glucosyltransferase activities (Fig. 4B). Although no collagen glycosyltransferase activity could be attributed to CEECAM1, we did confirm that the recombinant protein was expressed in Sf9 cells as were GLT25D1, GLT25D2, and LH3, as shown by SDS-PAGE (Fig. 4C). To confirm the identities of the GLT25D1, GLT25D2, CEECAM1, and LH3 proteins, the corresponding bands were excised from the gel, digested with trypsin, and submitted to tandem MS peptide sequencing (data not shown). However, even though it was shown to be expressed, it is still possible that the levels of CEECAM1 could be too low to detect activity.

***K_m* values.** The apparent K_m of GLT25D1 and GLT25D2 was determined for the bovine collagen type I acceptor and for UDP-Gal as a donor substrate, since these values had been reported previously for the semipurified ColGalT activity (23). The K_m values of GLT25D1 and GLT25D2 for the collagen type I acceptor were 13.6 g/liter and 9.8 g/liter, respectively (Fig. 5A and C), whereas Myllyla et al. reported a K_m of 150 g/liter for the partially purified chicken ColGalT enzyme. The K_m values for UDP-Gal were 18.77 μM for GLT25D1 and 33.53 μM for GLT25D2 (Fig. 5B and D). These values are comparable to those reported by Myllyla et al., who determined a K_m value for UDP-Gal of 30 μM for the partially purified chicken ColGalT (23).

ColGalT reaction products. The products of the GLT25D1- and GLT25D2-mediated ColGalT reactions were further analyzed to confirm the transfer of β-linked Gal to Hyl residues on collagen. The reaction products were hydrolyzed in 4 M NaOH to yield single amino acids. After derivatization with 9-fluorenylmethoxy carbonyl and separation by reverse-phase HPLC,

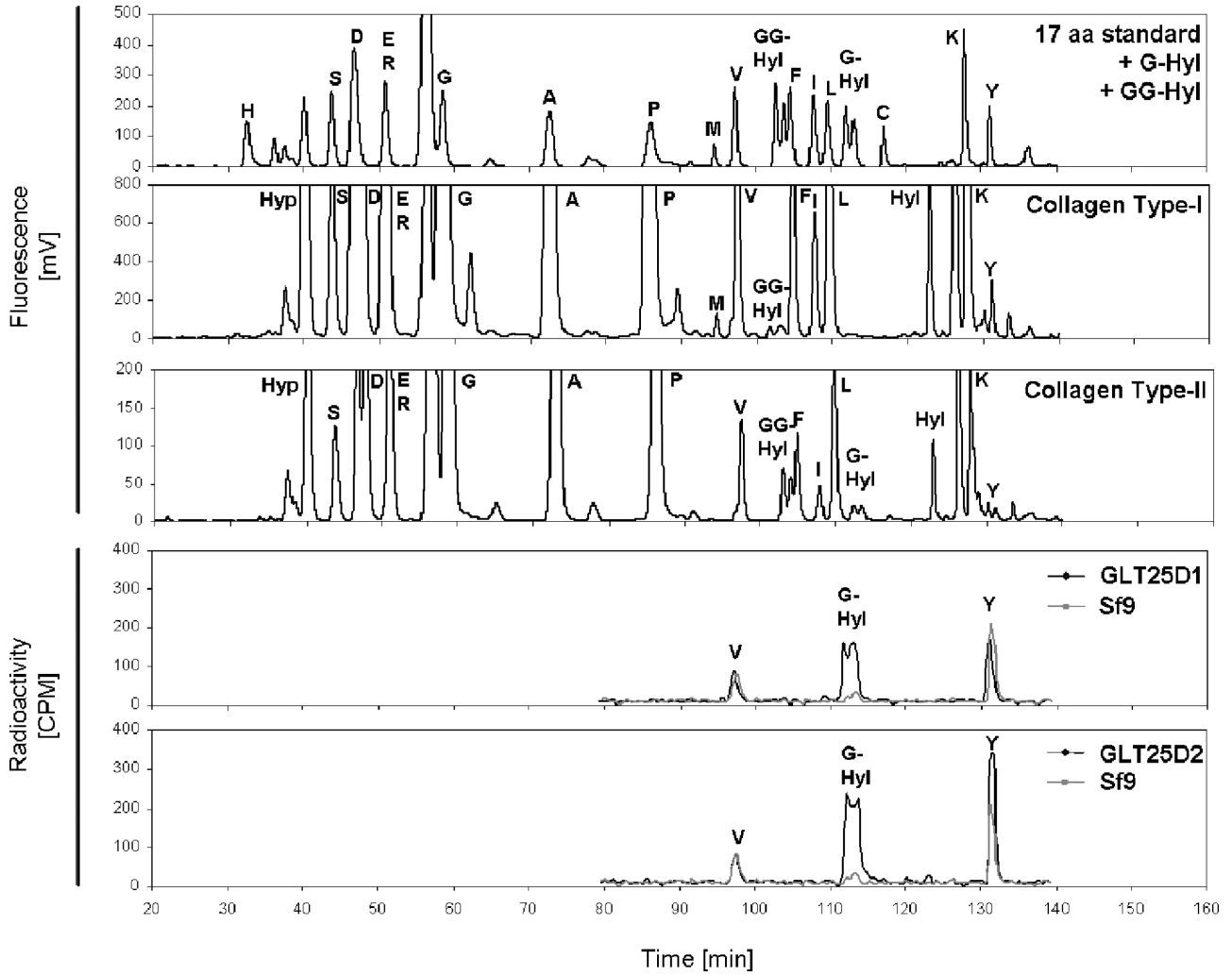

FIG. 6. Product identification by reverse-phase HPLC. The first panel represents an amino acid standard containing the standards for GHyl and GGHyl. The second and third panels show the amino acid profiles of bovine collagen type I and type II hydrolysates, respectively. The lower two panels show the radioactive trace obtained after reaction of collagen type I with GLT25D1 and GLT25D2. [^{3}H]Val and [^{14}C]Tyr were used as internal amino acid standards. Amino acids are marked in single-letter code. Hyp, hydroxyproline.

the amino acid profiles obtained from collagen type I, collagen type II, and the GLT25D1-, GLT25D2-reacted collagen acceptors were compared to a profile of authentic amino acid standards. The amounts of GHyl and GGHyl were higher in collagen type II than in collagen type I, as measured by the GHyl/Hyl and GGHyl/Hyl ratios, respectively (Fig. 6). This finding was in agreement with the values reported in the literature (16). The analysis of additional types of collagen, such as collagen type IV and sponge collagen, confirmed the variable extent of Hyl glycosylation across collagens (data not shown). The amino acid profiles obtained after GLT25D1 and GLT25D2 reactions in the presence of UDP-[^{14}C]Gal were further analyzed by β counting (Fig. 6, lower panel). The [^{14}C]Gal signal comigrated with the GHyl standard, indicating that GLT25D1 and GLT25D2 indeed represent true ColGalT enzymes. To demonstrate the β-linkage between Gal and Hyl, the GLT25D1 and GLT25D2 reaction products were digested with β-galactosidase. [^{14}C]Gal-Hyl was prepared by alkaline hydrolysis of the GLT25D1 and GLT25D2 reaction products, and samples of 25,000 cpm were incubated overnight with bovine testis β-galactosidase. More than 80% of the radioactivity was recovered as free [^{14}C]Gal after β-galactosidase digestion, which confirmed the β-configuration of the linkage catalyzed by the GLT25D1 and GLT25D2 enzymes (Table 2).

TABLE 2. β-Galactosidase digestion of GLT25D1 and GLT25D2 reaction products[a]

Enzyme	Incubation with β-galactosidase	GHyl input (cpm)	Amt of product (cpm)	
			GHyl	Gal
GLT25D1	+	25,000	3,780	19,376
GLT25D1	−	25,000	20,996	1,073
GLT25D2	+	25,000	3,528	20,362[a]
GLT25D2	−	25,000	20,313	1,029

[a] Each value represents the average for two independent experiments.

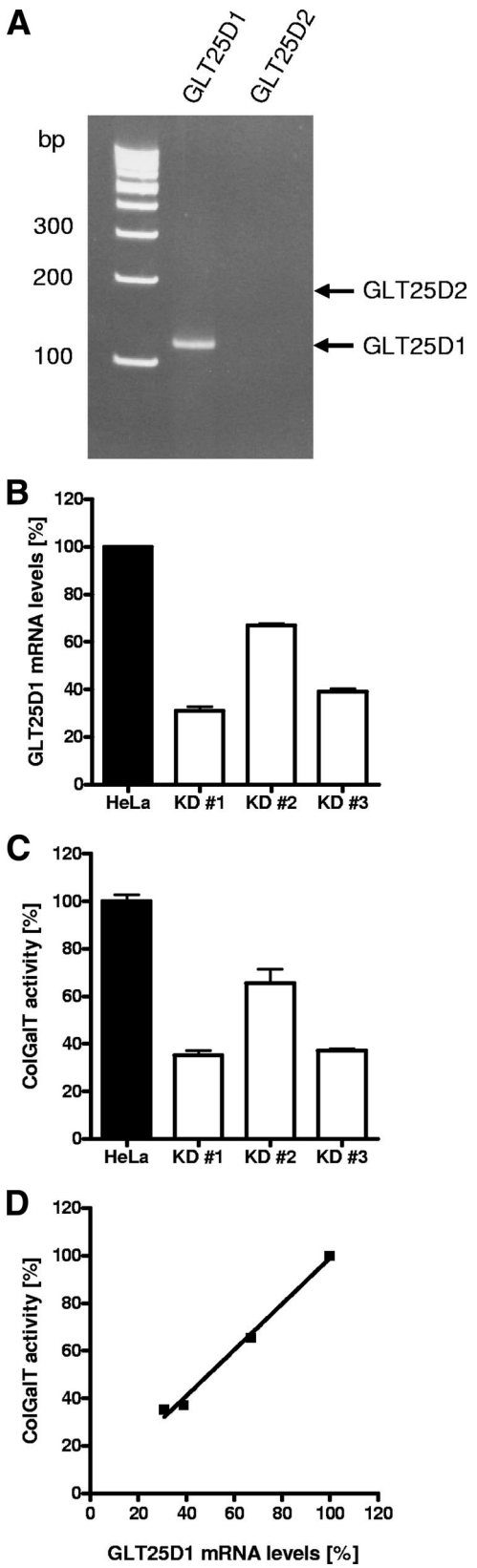

FIG. 7. Silencing of the *GLT25D1* gene. (A) RT-PCR detection of *GLT25D1* and *GLT25D2* expression in HeLa cells. (B) mRNA *GLT25D1* levels in wild-type HeLa cells (black bar) and in *GLT25D1*-silenced HeLa cells (KD #1 to KD #3, white bars). (C) Relative ColGalT activity in wild-type HeLa cells (black bar) and in *GLT25D1*-silenced HeLa cells (white bars). (D) Comparison between *GLT25D1* mRNA levels and ColGalT activity in wild-type HeLa cells (set to 100%) and those in *GLT25D1*-silenced HeLa cells.

ColGalT gene silencing. To correlate the expression of the GLT25D1 enzyme with endogenous ColGalT activity in human cells, we silenced the *GLT25D1* gene in HeLa cells by RNA interference. HeLa cells expressed only the *GLT25D1* gene, whereas *GLT25D2* expression remained undetected by RT-PCR (Fig. 7A). The *GLT25D1* gene was silenced by introducing short hairpin RNA constructs into HeLa cells. *GLT25D1* expression was reduced to 31%, 67%, and 39% of normal mRNA levels when expressing combinations of short hairpin RNA probes (Fig. 7B). The endogenous ColGalT activity of HeLa cells was measured and found to reach 5.7 pmol · min^{-1} mg $protein^{-1}$ in wild-type cells, whereas it was decreased to 2.0, 3.7, and 2.1 pmol · min^{-1} mg $protein^{-1}$ in *GLT25D1* knockdown cells. When the ColGalT activity of wild-type HeLa cells was normalized to 100%, this activity was decreased to 35%, 65%, and 37% in *GLT25D1* knockdown cells (Fig. 7C). The direct comparison between the level of *GLT25D1* expression and ColGalT activity in HeLa cells showed a strong correlation (Fig. 7D), thereby establishing a link between the GLT25D1 enzyme and ColGalT activity in vivo.

ColGalT gene expression. The expression of *GLT25D1*, *GLT25D2*, and *CEECAM1* in human tissues was analyzed by Northern blotting on 10 human tissues. A single *GLT25D1* transcript of 3.7 kb was detected in all tissues, whereas mRNA levels were highest in placenta, heart, lung, and spleen tissues (Fig. 8A). In contrast, the *GLT25D2* gene yielded a 5.1-kb mRNA that was detected only in brain and skeletal muscle at lower levels (Fig. 8A). A 2.3-kb *CEECAM1* transcript was detected in most tissues investigated, while a second transcript of 5.9 kb was also detected in brain tissue (Fig. 8A). The survey of *GLT25D1*, *GLT25D2*, and *CEECAM1* gene expression was extended to 75 human tissues and cell types using a Northern dot blotting array. The broad expression of the *GLT25D1* gene was confirmed in fetal and adult human tissues (Fig. 8B) (see Fig. S2 in the supplemental material). Similarly, the narrow range of *GLT25D2* gene expression was also evident throughout the RNA array (Fig. 8B) (see Fig. S2 in the supplemental material). The *CEECAM1* gene, which could not be related to ColGalT activity, showed widespread expression across tissues. Notably, *CEECAM1* was highly expressed in secretory tissues, such as salivary glands, pancreas, and placenta, and in the nervous system (Fig. 8B) (Fig. S2 in the supplemental material). Additionally, the *GLT25D1* and *CEECAM1* genes, but not *GLT25D2*, were also expressed in various carcinoma cell lines (see Fig. S2 in the supplemental material). This survey of ColGalT gene expression suggested that GLT25D1 represents the main source of ColGalT activity in human tissues while GLT25D2 appears to be specialized for a few cell types and possibly for a few collagen acceptors.

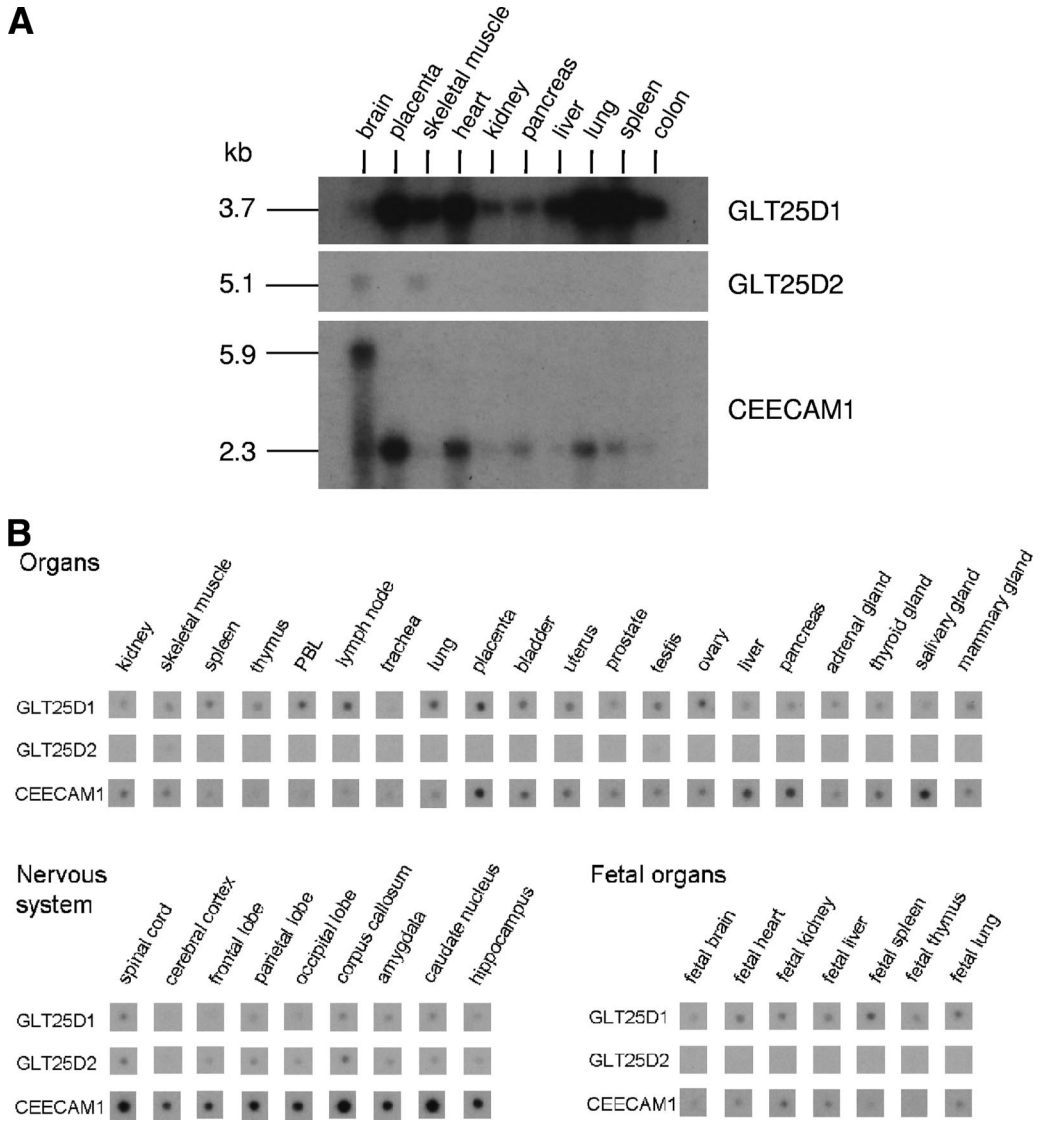

FIG. 8. Tissue Northern blotting. The mRNA expression patterns of *GLT25D1*, *GLT25D2*, and *CEECAM1* were analyzed in 10 human tissues (A) or in 36 human tissues and cell lines (B); a representative collection of additional tissues and cell types is shown in Fig. S2 in the supplemental material. PBL, peripheral blood leukocytes.

DISCUSSION

The identification of the GLT25D1 and GLT25D2 ColGalT enzymes and of the similar yet inactive relative CEECAM1 raises the question of a possible restricted specificity for collagen acceptor substrates. Previous work has shown that ColGalT recognizes collagen peptides of at least 500 to 600 Da and that Hyl alone is not a suitable acceptor (23, 31). The different activity levels of recombinant GLT25D1 and GLT25D2 enzymes toward the various types of collagen tested support the idea of a differential substrate recognition. The nature of the substrate recognition may be complex and may include glycosylated residues as part of the motifs recognized. In fact, GLT25D2 was more active toward native collagen type I and type II than toward the deglycosylated forms of these proteins (Table 1), which indicates that glycan chains somehow affected the recognition of the acceptor substrate by GLT25D2. Along the same lines, the apparent inactivity of CEECAM1 may be due to stringent structural requirements regarding the recognition of collagen peptides. A similarly complex mechanism of substrate recognition has been described for core glycosyltransferases acting on mucin proteins. Some members of the polypeptide *N*-acetylgalactosaminyltransferase family recognize peptide acceptors, including serine or threonine residues, near residues that were previously glycosylated by other *N*-acetylgalactosaminyltransferases (3). ColGalT assays with synthetic peptides including Hyl and GHyl at various positions will certainly answer the question of acceptor substrate recognition.

Alternatively, it is possible that CEECAM1 represents a ColGalT acting on a limited set of substrates. The screening of additional proteins, including collagen domains like adiponectin, the acetylcholine esterase complex COLQ, and the com-

plement protein C1q, might confirm this possibility. Finally, CEECAM1 may have lost any enzymatic activity over the course of evolution. In fact, CEECAM1 was first described as an adhesion protein (41) which might function as a carbohydrate-binding protein at the cell surface. However, the presence of the C-terminal RDEL motif would suggest that CEECAM1 is maintained in the ER.

The lysyl hydroxylase LH3 protein has been previously reported to possess three enzymatic activities, namely, a lysyl hydroxylase, a ColGalT, and a collagen glucosyltransferase activity (47). The glycosyltransferase activities attributed to LH3 were very low, casting doubt on their biological significance (27). The ColGalT activity of LH3 reported previously reached approximately twice the levels of endogenous ColGalT activity measured in Sf9 cells (47). It is possible that we could not distinguish the ColGalT activity of LH3 from the background activity levels in our assays. By comparison, the strong ColGalT activities described here for GLT25D1 and GLT25D2 implies that these proteins indeed represent true ColGalT enzymes. The dual glycosyltransferase activity of LH3 certainly requires closer attention, since it is expected that the catalysis of both β(1-O) and α(1-2) linkages would require distinct domains responsible for the retaining α(1-2) and inverting β(1-O) glycosyltransferase activities (26).

The identification of the *GLT25D1* and *GLT25D2* genes as encoding two ColGalT enzymes opens new ways to investigate the biological significance of collagen glycosylation. Genes similar to the human *GLT25D1* and *GLT25D2* genes can be found in all metazoan genomes sequenced to date. For example, the *Caenorhabditis elegans* gene *D2045.9* represents the probable ortholog of the human *GLT25D1* and *GLT25D2* genes. Knockdown of the *D2045.9* gene by RNA interference yields multiple abnormalities, such as deformed mating organs, slow growth, and uncoordinated locomotion (15, 36). By comparison, the loss of the lysyl hydroxylase gene *let-268* leads to a lethal phenotype associated with a defect in collagen type IV secretion (21, 25). The conservation of collagen glycosylation throughout animals and the essential role of collagen glycosylation in worms emphasize the importance of this modification. In humans, the *GLT25D1* and *GLT25D2* genes are found on human chromosome 19p13 and chromosome 1q25, respectively. The involvement of ColGalTs in the pathogenesis of connective tissue disorders linked to chromosomes 19p13 and 1q25, such as psoriasis (19) and epidermolysis bullosa (8, 18), can now be straightforwardly documented.

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