Biosynthesis of the Linkage Region of Glycosaminoglycans

CLONING AND ACTIVITY OF GALACTOSYLTRANSFERASE II, THE SIXTH MEMBER OF THE β1,3-GALACTOSYLTRANSFERASE FAMILY (β3GalT6)*

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A family of five β1,3-galactosyltransferases has been characterized that catalyze the formation of Galβ1,3GlcNAcβ and Galβ1,3GlcNAcβ linkages present in glycoproteins and glycolipids (ββGalT1, -2, -3, -4, and -5). We now report a new member of the family (ββ3GalT6), involved in glycosaminoglycan biosynthesis. The human and mouse genes were located on chromosomes 1p36.3 and 4E2, respectively, and homologs are found in Dro sophila melanogaster and Caenorhabditis elegans. Unlike other members of the family, ββ3GalT6 showed a broad mRNA expression pattern by Northern blot analysis. Although a high degree of homology across several subdomains exists among other members of the ββ3-galactosyltransferase family, recombinant enzyme did not utilize glucosamine- or galactosamine-containing acceptors. Instead, the enzyme transferred galactose from UDP-galactose to acceptors containing a terminal β-linked galactose residue. This product, Galβ1,3Galβ, is found in the linkage region of heparan sulfate and chondroitin sulfate (GlcβAβ,1,3Galβ,1,3Galβ,1,4Xylβ-O-Ser), indicating that ββ3GalT6 is the so-called galactosyltransferase II involved in glycosaminoglycan biosynthesis. Its identity was confirmed in vivo by siRNA-mediated inhibition of glycosaminoglycan synthesis in HeLa S3 cells. Its localization in the medial Golgi indicates that this is the major site for assembly of the linkage region.

Glycosaminoglycan biosynthesis initiates by the formation of the linkage tetrasaccharide, GlcβAβ,1,3Galβ,1,3Galβ,1,4Xylβ-O-Ser, which serves as a primer for the addition of alternating GlcNAc or GalNac and glucuronic acid (1). The assembly process involves the sequential transfer of monosaccharide residues from the corresponding nucleotide sugars starting at the reducing end. Xylosyltransferase (2), galactosyltransferase I (GalTI) (3, 4), and glucuronosyltransferase (GlcATI) (5, 6) have been cloned and partially characterized. In addition, mutant cell lines have been identified that contain defects in each of these enzymatic steps (7–9), and C. elegans mutants in GalTI (sqv3) and the GlcATI (sqv8) have been described, demonstrating the importance of these reactions and glycosaminoglycans in cellular processes and organismal development (10, 11). The enzyme that transfers the second galactose unit, galactosyltransferase II (GalTII) has not yet been identified. Previous studies have described the appropriate enzyme activity in tissue extracts (12), but the enzyme has not yet been purified or cloned. Presumably, GalTII should be distinct from GalTI based on linkage (β1,3 versus β1,4) and substrate differences (galactose versus xylose).

GalTII catalyzes the formation of Galβ1,3Gal linkage; therefore, it belongs to a family of β1,3-galactosyltransferases. Five members of this family have already been described (13–20), with a possible homolog in Drosophila (Broncia) (21). None of the ββ3GalT enzymes characterized to date have activity with galactose-terminated oligosaccharide acceptors, suggesting that another member of the family must exist in order to catalyze the formation of the linkage region of glycosaminoglycans.

In this report, we describe the identification of GalTII and show that it is the sixth member of confirmed β1,3-galactosyltransferases. This enzyme was actually identified in a previous publication but was designated a β1,3 N-acetylgalcosaminyltransferase because of a clerical error (22). Here we demonstrate that the expressed protein catalyzes the formation of Galβ1,3Gal linkages. The protein sequence is a close homolog of other β1,3-galactosyltransferase, and orthologs are present in Drosophila melanogaster and Caenorhabditis elegans genomes, consistent with the idea that this enzyme is broadly distributed as might be expected for an enzyme involved in glycosaminoglycan biosynthesis.

MATERIALS AND METHODS

Cell Culture—Chinese hamster ovary cells (CHO-K1), COS-7, and HeLa S3 cells were obtained from the American Type Culture Collect...
tion (ATCC CCL61, CRL-1651, and CCL2.2). CHO cells were grown under an atmosphere of 5% CO₂ in air and 100% relative humidity in Ham’s F-12 growth medium or F-12/Dulbecco’s modified Eagle’s medium (1:1, v/v) (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories), 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin G. COS-7 and HeLa S3 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal bovine serum and antibiotics.

Cloning and Expression of the Human and Mouse GalTII cDNAs—The human and mouse GalTII cDNAs were isolated from λ-phage libraries of human fetal brain cDNA (CLONTECH) and mouse newborn brain cDNA (Stratagene) using as a probe a PCR fragment derived from the expressed sequence tag AA150140. The PCR fragment was amplified from human genomic DNA by primers 5’-CCCTGCCCTACG-3’ and 5’-phage inserts were subcloned into the pFastbac1 vector (Life Technologies). Recombinant baculoviruses were generated by site-mediated transposition as recommended by the manufacturer. The full-length cDNAs flanked by EcoRI adaptors derived from the λ-phage inserts were subcloned into the pFastbac1 vector (Sigma). An XbaI fragment isolated from a partial human GalTII cDNA truncated after the transmembrane domain was ligated and expressed as a soluble secreted form using the pFLAG-CMV-1 vector (Sigma). An FLAG M2 (Sigma) and protein A-agarose beads (Amersham Pharmacia Biotech). The conditioned media and beads were mixed end-over-end overnight at 4 °C and centrifuged for 5 min, and the supernatant was aspirated. The beads were washed twice with 10 ml of 20% (v/v) glycerol in 50 mM Tris-HCl, pH 7.4, and resuspended in the same buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) to achieve a ~50% (v/v) slurry. The immobilized enzyme was stable at 4 °C for at least 4 months.

Northern Blot Analysis—GalTII mRNAs were detected by Northern blot analysis using commercially available multiple tissues poly(A)⁺ RNA blots (CLONTECH). A probe, derived from the human GalTII cDNA between nucleotides 616 and 983, was labeled and hybridized to RNA blots (CLONTECH). A probe, derived from the human GalTII cDNA between nucleotides 616 and 983, was labeled and hybridized to RNA blots (CLONTECH).

FIG. 1. ClustalX alignment of the human β3GalT family of proteins (GalT1, GenBank™ accession number E07739; GalT2, GenBank™ accession number Y15014; GalT4, GenBank™ accession number Y15061; GalT5, GenBank™ accession number AB020337).

### Table I

| Synthetic acceptor                  | Enzyme activity |
|-------------------------------------|-----------------|
| Monosaccharides (5 mM)               |                 |
| Xylβ-1,4O-Bn, -O-naphthol            | ND              |
| Galβ-1,3O-NM                         | 18              |
| Galβ-1,6O-NP                         | ND              |
| GalNAcβ-1,3O-NM, -O-4NP              | ND              |
| Disaccharides (5 mM)                 |                 |
| Galβ1,4Xylβ-1,3O-Bn                  | 84              |
| Galβ1,3GalNAcβ-1,3O-NM               | 6               |
| Galβ1,3Galβ-1,3O-NM                  | 1               |
| Galβ1,4GalNAcβ-1,3O-NM               | 4               |
| Galβ1,3GalNAcβ-1,6O-NM               | ND              |
| GalNAcβ1,3Galβ-1,3O-NM               | ND              |
| Man1,6Man-α-C10                      | ND              |
cells, the standard reaction (25 μl) contained 5 μl of protein A bead slurry (50%) containing immobilized enzyme, 40 mM MES, pH 6.0, 0.3 μCi of UDP-[1-3H]galactose (PerkinElmer Life Sciences), 150 μM UDP-galactose, 5 mM acceptor, 15 mM MnCl₄, and 50 mM KCl. After incubation at 37 °C for 1 h, the reaction products were diluted with 1 ml of 0.5 M NaCl and applied to a Sep-Pak C18 cartridge (100 mg; Waters). After washing the cartridge with 5 ml of water, the products were eluted with 50% methanol, dried, and counted by liquid scintillation.

**Product Identification**—Twenty small scale enzymatic assays were performed in parallel for 4 h using Galβ1,4XYβ-o-Bn as substrate and nonradioactive UDP-galactose as donor. After the clean up on a Sep-Pak C18 cartridge, the product was separated from the large amounts of unreacted disaccharide acceptor by silica gel chromatography using dichloromethane/methanol (100:3, v/v). Samples were analyzed on silica gel 60 aluminum-backed high performance thin layer chromatography plates (Merck). A section of the plate was stained with α-naphthyl reagent and heated at 110 °C for 5 min to detect the product (26).

The disaccharide substrate and purified trisaccharide product were repeatedly exchanged in D₂O (100%; Sigma) with intermediate lyophilization. The trisaccharide was dissolved in a final volume of 40 μl of 100% D₂O and transferred into a 40-μl nanocell. The disaccharide standard was dissolved in a final volume of 0.6–0.7 ml and transferred to a 5-mm tube (Wilmad 528-PP). ¹H NMR experiments were carried out on a Varian Unity Inova 500-MHz spectrometer at 25 °C. A SUN Microsystems Ultra-10 computer running Varian’s VNMR software (version 6.1B) controlled data acquisition. Chemical shifts are relative to 4,4-dimethyl-4-silapentane-1-sulfonate; they were typically measured relative to the residual acetate peak (δ 1.908 ppm at 22–30 °C and δ 6–8) and compared with literature values (27–31).

**Small Interfering RNA (siRNA) Inhibition of GalTII**—Two small interfering RNAs (siRNA GalTII-A and -B) were designed to target GalTII based on the method of Elbashir and co-workers (32). siRNA GalTII-A targeted nucleotides 461–482 numbered from the start codon (AY050570, sense: 5'-GGCCGCCAGCACGACACUCUUGTT-3', antisense: 5'-GAAGGAGUGCUGUCCGCTT-3'). siRNA GalTII-B targeted nucleotides 65-87 (sense: 5'-GACGCAGCGACCGUGCGCTT-3', antisense: 5'-GGCGCCACGGCGUGCTT-3'). As a control for nonspecific effects of siRNA, oligonucleotides were designed to target mouse lamin A (mLaminA) using nucleotides 260–280 (NM019390; sense: 5'-GAAGCAGCUUCAGGAUGAGAU-3', antisense: 5'-CUCAUGCGCAUGCGCUU-3'). siRNAs were obtained from Genset Oligos (Paris), and the complementary oligonucleotides were annealed as described previously (32). For each 30-mm culture dish of cells, 12 μl of the 20 μM stock siRNA duplex was mixed with 200 μl of Oligofectamine (Life Technologies, Inc.). This mixture was gently added to a solution containing 12 μl of Oligofectamine (Life Technologies, Inc.) in 48 μl of Opti-MEM. After 20 min at room temperature, 128 μl of Opti-MEM was added. This solution was gently overlaid onto 10–20% confluent HeLa S3 cells, which had been previously washed with Opti-MEM. After 5 h, 2 ml of Dulbecco’s modified Eagle’s medium with 30% fetal calf serum was added without removing the transfection media. Four days later, the cells were treated with trypsin and resuspended in 30-mm dishes. Three rounds of siRNA transfection were required to down-regulate glycosaminoglycan biosynthesis as determined by ³⁵SO₄ incorporation (33).

**Fluorescence Microscopy**—A green fluorescent protein (GFP)-fused form of GalTII was prepared by amplifying the full-length cDNA from the original pcDNA3.1(+ clone). An EcoRI restriction site at the 5'−end (5'-GCGGGAGTCTTATAAGGACCATCCC-3') and BamHI restriction site at the 3'-end (5'-CCGCGATCCCTCTCTCTCTGAGCCAC-3') were introduced. After digestion with the appropriate enzymes, the fragment was inserted in frame with GFP at the C terminus in pEGFP-N1. Cells grown on 24-well glass microscope slides were transfected with pEGFP-N1. After digestion with the appropriate enzymes, the fragment was inserted in frame with GFP at the C terminus in pEGFP-N1. Cells grown on 24-well glass microscope slides were transfected with pEGFP-N1. After digestion with the appropriate enzymes, the fragment was inserted in frame with GFP at the C terminus in pEGFP-N1. Cells grown on 24-well glass microscope slides were transfected with pEGFP-N1. After digestion with the appropriate enzymes, the fragment was inserted in frame with GFP at the C terminus in pEGFP-N1. Cells grown on 24-well glass microscope slides were transfected with pEGFP-N1. After digestion with the appropriate enzymes, the fragment was inserted in frame with GFP at the C terminus in pEGFP-N1. Cells grown on 24-well glass microscope slides were transfected with pEGFP-N1.
A large family of β1,3-galactosyltransferases has been described based on expressed sequence tags exhibiting a high degree of homology and by use of PCR-based cloning strategies (13–20). β3GalT1, -2, -3, and -5 utilize GlcNAc-terminated oligosaccharide as acceptor substrates, producing linkages characteristic of type 1 N-acetyllactosamine repeat units on N- and O-linked oligosaccharides and glycolipids, whereas β3GalT4 transfers galactose to the terminal GalNAc unit of the ganglioside series GM2, GD2, and GA2 acceptors (35). In addition to GlcNAcβ-based acceptors, β3GalT5 is also capable of transferring galactose to the terminal GalNAc unit of the globoside Gb4 (20). In a search for additional family members, we used the mouse β3GalT1, -2, and -3 (15) protein sequences to query the expressed sequence tag division of GenBank™, and the fragment AA150140 was identified. This fragment was used as a probe to isolate the corresponding full-length cDNAs from human and mouse libraries, which corresponded to open reading frames encoding proteins of 329 and 325 amino acids, respectively. This cDNA was previously associated with a β1,3-N-acetylgalosaminyltransferase activity (22), but the actual sequence responsible for the glucosaminyltransferase activity reported previously is encoded by AF092050 and AF092051. These correct designations have been posted in the GenBank™ sequence database. As shown below the cDNA actually encodes a β1,3-galactosyltransferase that participates in the formation of the linkage region of glycosaminoglycans. As the newest confirmed member of the β3GalT family, it is designated β3GalT6. However, in this paper it will be referred to as GAG GalTII after the nomenclature used in the proteoglycan field (1).

As shown in Fig. 1, GAG GalTII shows extensive homology to four other members of the human β1,3-galactosyltransferase family that have confirmed preferences for GlcNAc-terminated oligosaccharides. Like the other β1,3-galactosyltransferases, GAG GalTII has a typical type II transmembrane orientation. The tentative transmembrane domain consists of 19 amino acids starting at residue 12 from the N terminus. In the transmembrane domain, the GAG GalTII has a cysteine residue that is conserved in mouse, human, and C. elegans (Fig. 2). Interestingly, β3GalT1, β3GalT5, and the homologous Drosophila protein Brainiac also contain Cys residues in their putative transmembrane domains, but the function of these residues is unknown. One possibility is that they may affect the oligomerization of the protein as recently reported for α2,6-sialyltransferase I (36). The transmembrane domain is followed by a segment rich in Ser, Pro, Ala, and Gly, which has been described as the “SPLAG” domain in other transferases (37), and presumably represents a stem region that determines the distance between the catalytic domain and the membrane. All of the other motifs identified previously in the β3GalTs presumably define essential folds in the catalytic domain (13, 15, 22). These were also found in similar locations in GAG GalTII, but the conserved cysteine residues are either absent or located differently.

The chromosomal location of human and mouse GAG GalTII was determined using both the public genome data base and the partially annotated mouse genome. Human GalTII maps to chromosome 1p36.3 and occupies a similar position on the mouse chromosome at 4E2. Inspection of the sequence indicates that GalTII, like the other members of the β3GalTs, is encoded by a single exon in the human, but its organization in the mouse genome is unclear from the existing annotated data base. However, the gene appears to contain multiple exons in D. melanogaster and C. elegans. GalTII mRNA is expressed broadly across both human and mouse tissues (Fig. 3 and Ref. 22), revealing three different transcripts of ~1.6, 2.4, and 3.3 kilobases. This pattern differs from the expression profiles of the other β3GalTs, which tend to be restricted to specific tissues (13, 15, 16, 18, 19). The presence of multiple transcripts suggests that additional exons exist encoding 5′- and 3′-untranslated regions.

In nucleotide and amino acid sequences were used to measure the evolutionary relationship of GalTII and the other members of the β3GalT family (Fig. 4). Both dendrograms indicated a “starburst” relationship among the human β3GalT genes; i.e. all members of the family are relatively equidistant from each other (the internodal distances have high bootstrap support). This observation suggests that the β3GalT family emerged early in evolution and has not undergone recent duplications and radiations. Human GalTII has

**RESULTS AND DISCUSSION**

A large family of β1,3-galactosyltransferases has been described based on expressed sequence tags exhibiting a high degree of homology and by use of PCR-based cloning strategies (13–20). β3GalT1, -2, -3, and -5 utilize GlcNAc-terminated oligosaccharide as acceptor substrates, producing linkages characteristic of type 1 N-acetyllactosamine repeat units on N- and O-linked oligosaccharides and glycolipids, whereas β3GalT4 transfers galactose to the terminal GalNAc unit of the ganglioside series GM2, GD2, and GA2 acceptors (35). In addition to GlcNAcβ-based acceptors, β3GalT5 is also capable of transferring galactose to the terminal GalNAc unit of the globoside Gb4 (20). In a search for additional family members, we used the mouse β3GalT1, -2, and -3 (15) protein sequences to query the expressed sequence tag division of GenBank™, and the fragment AA150140 was identified. This fragment was used as a probe to isolate the corresponding full-length cDNAs from human and mouse libraries, which corresponded to open reading frames encoding proteins of 329 and 325 amino acids, respectively. This cDNA was previously associated with a β1,3-N-acetylgalosaminyltransferase activity (22), but the actual sequence responsible for the glucosaminyltransferase activity reported previously is encoded by AF092050 and AF092051. These correct designations have been posted in the GenBank™ sequence database. As shown below the cDNA actually encodes a β1,3-galactosyltransferase that participates in the formation of the linkage region of glycosaminoglycans. As the newest confirmed member of the β3GalT family, it is designated β3GalT6. However, in this paper it will be referred to as GAG GalTII after the nomenclature used in the proteoglycan field (1).

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undergone progressive variation in sequence, diverging as much from earlier phylogenetic forms as from other members in the family.

**Enzymatic Activity**—All of the previously described \( \beta \)\textsubscript{3}GalTs transfer galactose from UDP-galactose to glycans or glycoconjugates containing terminal \( \beta \)-linked GlcNAc or GalNAc residues. GAG GalTII, however, lacks this activity (Table I), and instead the recombinant enzyme prefers glycans with a terminal \( \beta \)-linked galactose residue or simple \( \beta \)-galactosides. In particular, GalTII reacted with Gal-1,4Xyl-O-Bn more strongly than any of the other substrates. A plot of activity versus acceptor concentration yielded an apparent \( K_m \) value of 5.7 mM (Fig. 5). The enzyme also showed a strict requirement for UDP-galactose, failing to transfer sugar from UDP-GalNAc and UDP-GlcNAc.

Structural characterization by one-dimensional \(^1\)H NMR of the enzymatic product formed using Gal-1,4Xyl-O-Bn as substrate showed that GAG GalTII forms a Gal-1,3Gal linkage (Table II and Fig. 6). Comparison of \(^1\)H NMR of the galactosylated product (Fig. 6B) and the substrate (Fig. 6A) showed the addition of a peak at \( \delta \) 4.605 \( (3J_{1,2} = 8.0 \) Hz) and 4.615 \( (3J_{1,2} = 7.8 \) Hz), indicative of an additional sugar residue in the \( \beta \)-configuration. Evidence of the newly formed Gal-1,3Gal linkage was evident from a shift in the Gal-2 H-4 peak at \( \delta \) 3.910 \( (3J_{3,4} = 3.0 \) Hz) in the acceptor to a peak at \( \delta \) 3.919 \( (3J_{3,4} = 3.0 \) Hz) in the product, confirming a Gal-1,3-linkage. These values were in good accordance with the corresponding L-serine glycoside (27–31).

**GalTII Is Required for Glycosaminoglycan Biosynthesis**—To determine whether GalTII was actually involved in glycosaminoglycan formation, siRNAs were generated to two different oligonucleotides segments of the human sequence. One round of transfection of HeLa cells only marginally inhibited glycosaminoglycan biosynthesis, as measured by \(^{35}\)SO\(_4\) incorporation.
biosynthesis. The cells were transiently transfected with the pEGFPN1-GalTII-GFP (green), α-mannosidase II (red), and the corresponding merged images (overlap indicated in yellow). D, E, and F show the localization of GalTII-GFP (green), CALNUC, cis-Golgi marker (red), and the corresponding merged images.

GalTII is localized to the medial Golgi—To determine the subcellular location of GAG GalTII, green fluorescent protein was fused to the C terminus, and the chimeric protein was expressed in Chinese hamster ovary cells (Fig. 8). By deconvolution microscopy, the tagged enzyme was detected in a punctate distribution in a perinuclear region overlapping to a greater extent with α-mannosidase II, a marker of the medial Golgi (36), than with CALNUC (nucleobindin), which has been localized to the cis-Golgi cisternae and cis-Golgi network (37). Prior studies indicate that GalTII (∂4GalT7) and glucuronosyltransferase I (the enzymes that generate the precursor for GalTII and that acts on its product, respectively) are localized similarly (40). Xylosyltransferases have recently been identified, but their localizations have not yet been established (2).

Immunochemical evidence suggests that this reaction occurs in an earlier endoplasmic reticulum compartment (41), suggesting that GAG synthesis initiates in the endoplasmic reticulum and then linkage region assembly is completed in the medial Golgi.

Conclusions—In this report, we have identified the cDNA for GAG GalTII and report that it is the previously described β,3,3-N-acetylgalcosaminyltransferase (22). This enzyme is part of a gene family that encodes at least six β,3,galactosyltransferases involved in glycoprotein, glycolipid, and proteoglycan processing. These enzymes in fact contain a motif in common with other β,3-galactosyltransferases. One of the enzymes, β3GalT3, has been shown to also transfer GalNAc in β,3, linkage to globosides (42), and four structurally related β,3,3-N-acetylgalcosaminyltransferases have been recently cloned based on sequence homology to β3GalT1 (43–45). In contrast to these enzymes, GAG GalTII (β3GalT6) preferentially acts on Galβ1,4Xyl, which is found in the linkage region of glycosaminoglycans. Furthermore, silencing of the gene by siRNA blocked glycosaminoglycan assembly in vitro, verifying the identity of this cDNA as GAG GalTII. Its localization to the medial Golgi along with GalTII and GlocATI is consistent with the idea that linkage region formation takes place in this compartment.

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REFERENCES
1. Esko, J. D., and Lindahl, U. (2001) J. Clin. Invest. 108, 169–173
2. Gotting, C., Kuhn, J., Zahn, R., Brinkmann, T., and Klessieke, K. (2000) J. Mol. Biol. 304, 517–528
3. Okajima, T., Yoshida, K., Kondo, T., and Furukawa, K. (1999) J. Biol. Chem. 274, 32915–32918
4. Almeida, R., Levery, S. B., Manel, U., Kresse, H., Schwientek, T., Bennett, E. P., and Clausen, H. (1999) J. Biol. Chem. 274, 26165–26171
5. Kitagawa, H., Tone, Y., Tamura, J., Neumann, K. W., Ogawa, T., Oka, S., Kawasaki, T., and Sugahara, K. (1998) J. Biol. Chem. 273, 6615–6618
6. Wei, G., Bai, X. M., Sarkar, A. K., and Esko, J. D. (1999) J. Biol. Chem. 274, 7857–7864
7. Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3197–3201
8. Esko, J. D., Weinke, J. L., Taylor, W. H., Ekborg, G., Reden, L., Anantharamaiah, G., and Gawish, A. (1987) J. Biol. Chem. 262, 12189–12195
9. Bai, X. M., Wei, G., Sinha, A., and Esko, J. D. (1999) J. Biol. Chem. 274, 13017–13024
10. Herman, T., and Horvitz, H. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 974–979
11. Boulis, D. A., Wei, G., Toyoda, H., Kinoshita-Toyoda, A., Waldrip, W. R., Esko, J. D., Robbins, P. W., and Selleck, S. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10838–10843
12. Helting, T., and Roden, L. (1989) J. Biol. Chem. 264, 2790–2798
13. Amado, M., Almeida, R., Carneiro, F., Levery, S. B., Holmes, E. H., Nomoto, M., Hollingsworth, M. A., Hassan, H., Schwientek, T., Nielsen, P. A., Bennett, E. P., and Clausen, H. (1998) J. Biol. Chem. 273, 12770–12778
14. Amado, M., Almeida, R., Schwientek, T., and Clausen, H. (1999) Biochim. Biophys. Acta Gen. Subj. 1451, 35–53
15. Hennet, T., Dinter, A., Kuhnert, P., Mattu, T. S., Ruddle, P. M., and Berger, E. G. (1998) J. Biol. Chem. 273, 58–65
16. Kolbinger, F., Streiff, M. B., and Katsopidis, A. G. (1998) J. Biol. Chem. 273, 433–440
17. Salvini, R., Bardoni, A., Valli, M., and Trinchera, M. (2001) J. Biol. Chem. 276, 3564–3573
18. Zhou, D., Berger, E. G., and Hennet, T. (1999) Eur. J. Biochem. 263, 571–576
19. Ishikii, S., Togayachi, A., Kudo, T., Nishihara, S., Watanabe, M., Kubota, T., Kitajima, M., Shiraishi, N., Sakai, K., Andoh, T., and Narimatsu, H. (1999) J. Biol. Chem. 274, 12499–12507
20. Zhou, D., Henion, T. R., Jungalwala, F. B., Berger, E. G., and Hennet, T. (2000) J. Biol. Chem. 275, 22631–22634
21. Yoon, Y. P., Schultz, J., Gledek, M., and Bork, P. (1997) Cell 88, 9–11
22. Zhou, D., Dinter, A., Gutierrez Gallego, R., Kamerling, J. P., Vliegenthart, J. F., Berger, E. G., and Hennet, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 406–411; Correction (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11673–11675
23. Sarkar, A. K., Brown, J. R., and Esko, J. D. (2000) Carbohydr. Res. 328, 287–300
24. Leguenfa, P. N., Sarkar, A. K., and Esko, J. D. (1996) J. Biol. Chem. 271, 19159–19165
25. Brown, J. R., Field, R. A., Barker, A., Guy, M., Grewal, R., Kho, K. H., Brennan, P. J., Besra, G. S., and Chatterjee, D. (2001) Bioorg. Med. Chem. 9, 815–824
26. Siakotos, A. N. (1965) J. Am. Oil Chem. Soc. 42, 913–919
27. Wiersuzsksi, J. M., Michalski, J. C., Montreuil, J., Strecker, G., Peter-Katalinic, J., Egge, H., van Halbeek, H., Mutsaers, J. H., and Vliegenthart, J. F. (1997) J. Biol. Chem. 272, 6650–6657
28. Van Halbeek, H., Dorland, L., Veldink, G. A., Vliegenthart, J. F., Garegg, P. J., Norberg, T., and Lindberg, B. (1992) Eur. J. Biochem. 127, 1–6
29. Krishna, N. R., Choe, B.-Y., Prabhakaran, M., Ekborg, G. C., Roden, L., and

FIG. 7. siRNA silencing of GalTII inhibits glycosaminoglycan biosynthesis. HeLa cells were transiently transfected three consecutive times with siRNA GalTII-A, GalTII-B, or mLaminA duplexes (see “Material and Methods”). The cells were labeled briefly with [35S]SO4 and the extent of [35S]glycosaminoglycan synthesis was determined.

FIG. 8. Localization of GFP·GalTII chimeric protein. Chinese hamster ovary cells were transiently transfected with the pEGFPN1-GalTII construct and imaged by deconvolution microscopy (see “Materials and Methods”). A, B, and C show the localization of GalTII-GFP (green), α-mannosidase II (red), and the corresponding merged images (overlap indicated in yellow). D, E, and F show the localization of GalTII-GFP (green), CALNUC, cis-Golgi marker (red), and the corresponding merged images.
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Harvey, S. C. (1990) *J. Biol. Chem.* **265**, 18256–18262

30. Agrawal, P. K., Jacquinet, J. C., and Krishna, N. R. (1999) *Glycobiology* **9**, 669–677

31. Krishna, N. R., and Agrawal, P. K. (2000) *Adv. Carbohydr. Chem. Biochem.* **56**, 201–234

32. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2000) *Nature* **411**, 494–498

33. Bame, K. J., and Esko, J. D. (1989) *J. Biol. Chem.* **264**, 8059–8065

34. Dulbecco, R., and Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182

35. Miyazaki, H., Fukumoto, S., Okada, M., Hasegawa, T., and Furukawa, K. (1997) *J. Biol. Chem.* **272**, 24794–24799

36. Qian, R., Chen, C., and Colley, K. J. (2001) *J. Biol. Chem.* **276**, 28641–28649

37. Shworak, N. W., Liu, J. A., Petros, L. M., Zhang, L. J., Kobayashi, M., Copeland, N. G., Jenkins, N. A., and Rosenberg, R. D. (1999) *J. Biol. Chem.* **274**, 5170–5184

38. Velasco, A., Hendricks, L., Moremen, K. W., Tulsiain, D. R., Touster, O., and Farquhar, M. G. (1993) *J. Cell Biol.* **122**, 39–51

39. Lin, P., Le-Niculescu, H., Hofmeister, R., McCaffery, J. M., Jin, M., Hennemann, H., McQuistan, T., De Vries, L., and Farquhar, M. G. (1998) *J. Cell Biol.* **141**, 1515–1527

40. Pinhal, M. A. S., Smith, B., Olsen, S. K., Aikawa, J., Kimata, K., and Esko, J. D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 12984–12989

41. Vertel, B. M., Walters, L. M., Flay, N., Kearns, A. E., and Schwartz, N. B. (1993) *J. Biol. Chem.* **268**, 11105–11112

42. Okajima, T., Nakamura, Y., Uchikawa, M., Haslam, D. B., Namata, S. I., Furukawa, K., Urano, T., and Furukawa, K. (2000) *J. Biol. Chem.* **275**, 40498–40503

43. Shiraishi, N., Natsume, A., Togayachi, A., Endo, T., Akashima, T., Yamada, Y., Imai, N., Nakagawa, S., Koizumi, S., Sekine, S., Narimatsu, H., and Sasaki, K. (2001) *J. Biol. Chem.* **276**, 3488–3507

44. Togayachi, A., Akashima, T., Okubo, R., Kudo, T., Nishihara, S., Iwasaki, H., Natsume, A., Mio, H., Inokuchi, J., Irimura, T., Sasaki, K., and Narimatsu, H. (2001) *J. Biol. Chem.* **276**, 22032–22040

45. Henion, T. R., Zhou, D., Wolfer, D. P., Jungalwala, F. B., and Hennet, T. (2001) *J. Biol. Chem.* **276**, 30261–30269