An Amino Acid Region at the N-Terminus of Rat Hepatoma α1→2 Fucosyltransferase Modulates Enzyme Activity and Interaction with Lipids: Strong Preference for Glycosphingolipids Containing Terminal Galβ1→3GalNAc-Structures†

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Received January 31, 2001

ABSTRACT: A GDP-fucose:GM_1 α1→2 fucosyltransferase (FucT) is induced during early stages of chemical hepatocarcinogenesis in parenchymal cells of Fischer 344 rats fed a diet supplemented with 0.03% *N*-2-acetylaminofluorene (AAF). This enzyme is undetectable in normal rat liver tissues but is highly expressed in many rat hepatoma cell lines, including rat hepatoma H35 cells. Enzymatic properties and acceptor specificity of native rat hepatoma H35 cell α1→2FucT, expressed recombinant full-length H35 cell α1→2FucT, and a truncated form missing the first 27 amino acid residues from the N-terminus, comprising the cytoplasmic and transmembrane domains of the enzyme, were studied. The results indicate that the recombinant full-length enzyme has a specific activity over 80-fold higher than the truncated enzyme. Both the native and recombinant full-length enzymes display significant activity in the absence of detergent or phospholipid and optimal activity in the presence of Triton CF-54 detergent. The truncated enzyme is optimally activated by CHAPSO, showing little activity in its absence. These findings are in agreement with previous studies demonstrating a requirement of a lipidic environment for optimal activity with this enzyme and suggest that the N-terminal transmembrane domain is important either in the maintenance of an active conformation or in allowing efficient interaction with acceptor glycolipids. Both the full-length and truncated enzymes transfer fucose not only to GM_1 and asialo-GM_1 (Gg_4) but also to galactosyl globoside (Gb_5) as well. Weak or undetectable transfer to lacto- and neolacto-series acceptors was observed, demonstrating a strong preference for terminal Galβ1→3GalNAc- structures. The structures of two reaction products generated by expressed recombinant full-length α1→2FucT, which are known to be important tumor-associated antigens (fucosyl-GM_1 and fucosyl-Gb_5), were unambiguously confirmed by ^{1}H-NMR spectral analysis.

Rat hepatoma cell lines have been shown to have a high incidence of induction of synthesis of fucose-containing gangliosides (*1*, *2*). Further, the expression of the ganglio-B determinant ($II^3NeuAcIV^3\alpha GalIV^2FucGg_4$)[1] has been reported to occur during early stages of chemical carcinogenesis in rat liver following treatment with AAF (*3*, *4*). The induction of synthesis of this structure is an early event occurring in association with AAF-induced chemical carcinogenesis giving rise to a premalignant or malignant marker. This carbohydrate structure is the product of a normally unexpressed ganglioside GM_1-specific α1→2 fucosyltransferase (FucT) that is not expressed in normal rat liver tissue or hepatocytes but is activated in liver hepatocytes as a result of a carcinogenic stimulus and not by hepatotoxicity or liver regeneration (*5*, *6*). Upon activation of this enzyme, the fucosyl-GM_1 product then becomes an effective acceptor for naturally occurring α1→3 galactosyltransferase, leading to the appearance of a ganglioside with the same determinant structure as blood group B.

† This work was supported in part by Grant RO1 CA70740 from the National Cancer Institute (to E.H.H.) and by Grant NIH 5 P41 RR05351 from the National Institutes of Health Resource Center for Biomedical Complex Carbohydrates (to S.B.L.).

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[1] Abbreviations: FucT, fucosyltransferase; Fuc, L-fucose; AAF, *N*-2-acetylaminofluorene; ^{1}H-NMR, proton nuclear magnetic resonance; RT-PCR, reverse transcriptase polymerase chain reaction; GFP, cycle 3 green fluorescent protein; TBS-T, Tris-buffered saline−Tween (25 mM Tris, 0.137 M NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 7.4); DTE, dithioerythritol; HPTLC, high-performance thin-layer chromatography; IPA, isopropyl alcohol; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; Cer, ceramide. Glycolipids are abbreviated according to the recommendations of the IUPAC−IUB Commission on Biochemical Nomenclature (*63*), but the suffix -OseCer is omitted. The major glycosphingolipids referred to in this study are as follows: GM_1, $II^3NeuAcGg_4$, Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glcβ1→1Cer; fucosyl-GM_1, $II^3NeuAcIV^2FucGg_4$, Fucα1→2Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glcβ1→1Cer; ganglio-B, $II^3NeuAcIV^3\alpha GalIV^2FucGg_4$, Galα1→3[Fucα1→2]Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glcβ1→1Cer; Gb_5, galactosyl globoside, Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer; globo-H, fucosylgalactosyl globoside, V^2FucGb_5, Fucα1→2Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4 Glcβ1→1Cer; nLc_4, lacto*neo*tetraosylceramide, Galβ1→4GlcNAcβ1→3 Galβ1→4Glcβ1→1Cer; Lc_4, lactotetraosylceramide, Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; Lc, lactosylceramide, Galβ1→4Glcβ1→1Cer.

10.1021/bi0102104 CCC: $20.00
Published on Web 04/20/2001

Genes for several α1→2FucTs have been cloned from several species (*7*−*13*) and include both the secretor and H enzymes. The H enzyme preferentially fucosylates type 2 precursors, while the Se enzyme preferentially reacts with type 1 and 3 acceptors (*14*−*16*). Amino acid sequence alignments indicate that the rat hepatoma H35 cell GDP-fucose:GM_1 α1→2FucT gene is most homologous to the human *Sec2* gene (*17*). Unlike most other α1→2FucTs, which readily transfer fucose onto lacto-series core structures to produce the H structural precursor of blood group A or B antigens normally expressed on human erythrocytes, the rat hepatoma enzyme has been found to preferentially fucosylate ganglio-series structures, transferring fucose only weakly to lacto- or neo-lacto-series core structures (*17*−*19*). It is also inactivated by most detergents (*19*).

Since this particular α1→2FucT demonstrates high specificity for ganglio-series acceptors, we hypothesized that there might be a strong preference for the terminal Gal*β*1→3GalNAc- structure as found not only on ganglioside GM_1 and asialo-GM_1 but also on galactosyl globoside (Gb_5) as well. In the present study, we synthesized galactosyl globoside (Gb_5) and demonstrated that it too is an efficient acceptor for the rat hepatoma H35 cell α1→2FucT. Two of these structures, fucosyl-GM_1 and fucosylgalactosyl globoside (globo-H), were unambiguously confirmed by ^{1}H-NMR spectral analysis. While there is a strong preference for terminal Gal*β*1→3GalNAc- structures, full-length recombinant rat hepatoma α1→2FucT also demonstrated weak transfer of fucose to type 1 and 2 lacto- and neolacto-series acceptors, similar to the native enzyme.

In a previous report, we described the cloning and expression of a truncated rat hepatoma H35 cell α1→2FucT cDNA construct which represents the 353 amino acid catalytic domain of the enzyme (*17*). We have subsequently cloned and expressed the full-length α1→2FucT cDNA, which includes 27 additional amino acids at the N-terminus. The results indicate this 27 amino acid transmembrane/cytoplasmic region plays an important role in modulating the activity and behavior of the rat hepatoma α1→2FucT enzyme. The full-length enzyme has a much higher specific activity and is influenced to a far greater extent by its lipidic environment than the truncated form.

MATERIALS AND METHODS

Materials. Rat hepatoma H35 cells and simian COS-7 cells were obtained from the American Type Cell Collection (Rockville, MD). AAF was from Aldrich Chemical Co. (Milwaukee, WI). FITC-labeled goat antibodies to rabbit immunoglobulins were obtained from Dako (Denmark). RNAzol B total RNA isolation kit was from Tel-Test, Inc. (Friendswood, TX). Plasmids pcDNA3 and pcDNA3.1/NT-GFP-TOPO were from Invitrogen (San Diego, CA), and pPROTA was received from Dr. Bruce Macher (San Francisco State University, San Francisco, CA). Rabbit IgG−Sepharose 6 Fast Flow beads were from Amersham Pharmacia Biotech AB (Uppsala, Sweden), and DEAE-Dextran was obtained from Sigma (St. Louis, MO). rEGFP peptide standards, primary rabbit anti-GFP antibody, and secondary horseradish peroxidase-conjugated rabbit IgG secondary antibody were from Clontech (Palo Alto, CA). PCR primers were made on a Beckman Oligo 1000 synthesizer or were obtained from Integrated DNA Technologies (Coralville, IA). GDP-[^{14}C]fucose and [α-^{35}S]dATP were obtained from Dupont NEN (Boston, MA). DNA sequencing was done using the Sequenase Version 2.0 DNA sequencing kit from United States Biochemical Corp. (Cleveland, OH) or the SequiTherm EXCEL II DNA sequencing kit from Epicenter Technologies (Madison, WI). Final confirmatory vector sequencing was performed by Thetagen Genetic Diagnostic Services (Seattle, WA). All other reagents were of the highest quality commercially available.

Tissue Localization Studies. Liver samples were obtained from the caudate lobes of male Fischer 344 rats which had been fed Wayne Laboratory diet supplemented with 0.03% (w/w) AAF over a period of 3−5 weeks or control animals fed the same diet without AAF as previously described (*6*) and prepared for cryostat sectioning. The sections were initially fixed in 4% paraformaldehyde for 20 min and then blocked by incubation with PBS containing 5% whole goat serum for 1 h to reduce nonspecific antibody binding. The sections were incubated overnight with a 1:10 dilution of serum containing polyclonal rabbit antibody specific for the ganglio-B determinant ($II^3NeuAcIV^3\alpha GalIV^2FucGg_4$) prepared as previously described (*6*), washed extensively with PBS containing 5% whole goat serum, and then incubated again with 1:40 FITC-labeled secondary goat anti-rabbit whole Ig (Dako) for 1 h. The PBS-washed sections were mounted with aqua-mount and examined by fluorescent microscopy. Control sections in which the primary antibody incubation was omitted were consistently negative.

RT-PCR Analysis of Normal and AAF-Fed Rat Liver Tissue for the Presence of α1→2 Fucosyltransferase. Total RNA was extracted from approximately 200 mg of normal, healthy Fischer 344 rat liver tissue and 200 mg of liver tissue from rats fed 0.03% AAF (w/w) for 5 weeks using the RNAzol B method (Tel-Test, Inc., Friendswood, TX). RT-PCR was conducted as previously described (*17*) using 200 pM α1→2FucT-specific primers, (forward) 5′-GGC-CGCTTTGGGAACCAGATGG-3′ and (reverse) 5′-GGT-TACACTGCGTGAGCAGCGC-3′, which have been found to reproducibly yield a single PCR product of 0.6 kb from rat hepatoma H35 cell total RNA.

Cell Culture. Rat hepatoma H35 cells and simian COS-7 cells were grown in tissue culture plates in RPMI 1640 and Dulbecco's modified Eagle's medium (DME), respectively, supplemented with 10% fetal calf serum. These were harvested and passed 1:4 every 5−6 days.

Construction of the Full-Length Rat α1→2FucT Expression Vector and Expression of α1→2FucT cDNA in COS-7 Cells. The cloning and expression of a truncated rat hepatoma H35 α1→2FucT cDNA construct (CAT-RFT) which represents the 353 amino acid catalytic domain of the enzyme inserted into pPROTA plasmid for protein A:α1→2FucT fusion protein has been described (*17*). Thus, the majority (1059 bp) of the rat hepatoma H35 cell α1→2FucT sequence was already known. On the basis of information obtained from a 2984 bp *Rattus norvegicus* FTB mRNA sequence (Accession Number AB006138) found in GenBank databases (*20*), a forward primer was designed from the putative start of translation, determined by the rules of Kozak (*21*). This mRNA was found to contain 213 nucleotides of upstream untranslated sequence and over 1580 bp of 3′ untranslated sequence. As it is reported in the GenBank database, this

A

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atggccagcgcccaggttcctttctcctttcctctggcccacttcctcatctttgtcttcgtgacttccaccatcat
ccacctccagcagcgaatagtgaagctccaacccctgtcagagaaggaattaccgatgacgactcaaatgtcctcgg
gaaacacagaaagcccagagatgcgacgggacagcgagcagcatgggaatggagagctgcggggcatgttcacgatc
aattccattggccggctggggaaccagatgggcgaatacgccacactctttgcactggccaggatgaacggacggct
tgcgttcatccccgcatccatgcacaacgctctagcgcccatcttcaggatcagcctcccggtgttacacagcgaca
cggccaaaaagatcccatggcagaattaccatctcaacgactggatggaggagcgttaccgccacattccgggacac
tttgtgcgcttcacgggatacccgtgctcctggaccttctaccaccacctgcgcccagagatcctgaaggagttcac
cctgcatgaccacgtgcgggaggaggcccaggccttcctgcgtggtctgcgggtgaatgggagccagccgagtactt
ttgtgggtgtccatgtgcgccgaggggactatgtgcatgtcatgcctaatgtgtggaagggcgtggtggctgaccgg
ggttacctggaaaaggccctggatatgttccgggcacgctattcatctccagtcttcgtggttacaagcaacggtat
ggcctggtgccgggagaacattaatgcttcccgaggagacgtggtgttcgcgggcaatggtattgaggggtcgccag
ccaaggacttcgcgctgctcacccagtgcaaccacaccatcatgactattgggacctttgggatttgggctgcctac
ctggcaggtggtgataccatctacttagccaactacacccttccggattctccgttcctcaaagtctttaagccaga
ggcagccttcctacccgaatgggtgggcatccctgccgatctgtccccactccttaaggcattaacaccagcctgtc
ctcggtcccacttccacctcaaggcaaaaggagtcacttgttacgtcgcaggaagagccttctgatg
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B

MASAQVPFSFPLAHFLIFVFVTSTIIH LQQRIVKLQPLSEKELPMTTQMSSGNTESPEMRRDSEQHGNGELR
GMFTINSIGRLGNQMGEYATLFALARMNGRLAFIPASMHNALAPIFRISLPVLHSDTAKKIPWQNYHLNDWMEERYRH
IPGHFVRFTGYPCSWTFYHHLRPEILKEFTLHDHVREEAQAFLRGLRVNGSQPSTFVGVHVRRGDYVHVMPNVWKGVV
ADRGYLEKALDMFRARYSSPVFVVTSNGMAWCRENINASRGDVVFAGNGIEGSPAKDFALLTQCNHTIMTIGTFGIWA
AYLAGGDTIYLANYTLPDSPFLKVFKPEAAFLPEWVGIPADLSPLLKALTPACPRSHFHLKAKGVTCYVAGRAF

FIGURE 1: Nucleotide and deduced amino acid sequence of the full-length rat hepatoma H35 cell α1→2 fucosyltransferase. (A) Nucleotide sequence of a 1140 bp RT-PCR product representing the entire coding region of rat hepatoma H35 cell α1→2FucT. This nucleotide sequence has been deposited in GenBank under Accession Number AF264005. (B) Deduced amino acid sequence of the full-length rat hepatoma H35 cell α1→2FucT gene. The 27 amino acids in boldface at the N-terminus represent the transmembrane/stem region of the enzyme.

particular sequence has an error at amino acid 354 (A→H) immediately followed by a nonsense mutation (TAA) for premature termination and, therefore, would not express an active enzyme. However, on the basis of the correct sequence information at the 5′ end, a forward primer was designed: 5′-GCCATGGCCAGCGCCCAGGTTCCT-3′. This primer was used in conjunction with a reverse primer, 5′-TCCCATCAGAAGGCTCTTCCTGC-3′, described previously in the construction of CAT-RFT (*17*) to RT-PCR the entire 1140 bp α1→2FucT coding region from rat hepatoma H35 cell total RNA (results not shown). This PCR product was sequenced (Figure 1) and determined to be the α1→2FucT associated with malignant transformation in rat liver cells (GenBank Accession Number AF264005). Full-length α1→2FucT cDNA was then cloned into the pcDNA3 vector (Invitrogen) in both the positive [FL-RFT(+)] and negative [FL-RFT(−)] orientations. These constructs in addition to the CAT-RFT plasmid were transiently transfected into COS-7 cells by the DEAE-dextran method (*22*). Cells were transfected with FL-RFT(+) and FL-RFT(−) or were harvested 4−5 days later for α1→2FucT assays. Secreted fusion protein from COS-7 cells transfected with CAT-RFT was purified from the conditioned medium of cells after 4−5 days on IgG−agarose beads as previously described (*23*).

Cloning into the pcDNA3.1/NT-GFP-TOPO Vector and Western Blot Analysis. Full-length and truncated α1→2FucT inserts generated by PCR were also cloned into pcDNA3.1/NT-GFP-TOPO plasmid (Invitrogen) for cycle 3 GFP fusion protein expression at the amino terminus of each enzyme. These constructs, GFP/FL-RFT and GFP/CAT-RFT, were used solely for the determination of specific activity of the full-length and truncated α1→2FucT enzyme forms. These plasmids were transfected as previously described into COS-7 cells and assayed for α1→2FucT activity. Comparable amounts of total protein from each transfected COS-7 cell lysate, determined by Bradford assays using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), along with rEGFP peptide standards (Clontech) were run on 10−20% Tricine gels (Novex, San Diego, CA) for Western blot analysis. Blots were screened for expression of cycle 3 GFP by autoradiography following incubation with primary rabbit anti-GFP antibody (1:100 in TBS-T), secondary horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:8000 in TBS-T), and Luminol western blotting reagent (Santa Cruz Biotech, Santa Cruz, CA). Bands representing cycle 3 GFP/α1→2FucT fusion protein were quantified by densitometry using an IS-1000 digital imaging system (Alpha Innotech Corp.) for specific activity determinations.

α1→2 Fucosyltransferase Assays. Standard assays of α1→2 fucosyltransferase activity were performed as previously described (*24*) in a reaction mixture volume of 0.1 mL containing 2.5 μmol of HEPES buffer, pH 7.2, 30 μg of GM_1 ganglioside acceptor substrate, 1 μmol of $MnCl_2$, 0.5 mM ATP, 15 nmol of GDP-[^{14}C]fucose (15 000 cpm/nmol), and enzyme from one of four sources: (1) pPROTA-expressed (CAT-RFT) enzyme bound to IgG−Sepharose beads, (2) expressed recombinant enzyme from homogenates of membranes from COS-7 cells transfected with pcDNA3 plasmids containing full-length α1→2FucT cDNA [FL-RFT-(+)], (3) expressed recombinant truncated and full-length GFP:α1→2FucT fusion protein enzyme from homogenates of membranes from COS-7 cells transfected with GFP/FL-RFT or GFP/CAT-RFT, and (4) native enzyme from H35 cell membrane homogenates. Membrane-bound enzyme homogenates were made by sonicating 1 volume of packed cells in 1.5 volume of buffer containing 1.0 M Hepes, pH 7.2, 25% glycerol, and 1 mM DTE for 2−3 min on ice in a sonic bath. In some cases, homogenates of membranes from untransfected COS-7 cells were included in the assays of CAT-RFT beaded enzyme. Construction of an H35 cell truncated α1→2FucT cDNA in the pPROTA vector (*25*) has been described (*17*). Expression of this plasmid, CAT-RFT, results in the production of a fusion protein composed of the protein A−IgG binding domain and the α1→2FucT catalytic domain. The expressed protein, a truncated version of the full-length enzyme, is conveniently isolated by binding

to IgG−Sepharose beads which can be directly assayed for enzyme activity (*26*). The reaction mixtures were incubated for 1 h at 37 °C, terminated by the addition of 0.1 mL of $CHCl_3$:CH_3OH (2:1), streaked onto a 4 cm wide strip of Whatman 3 paper, and developed with water overnight. After the papers were dried, the labeled product was extracted from the origins with two 5 mL washes of $CHCl_3$:CH_3OH:H_2O (10:5:1). The combined eluates were concentrated to dryness by an N_2 stream and dissolved in 20 μL of $CHCl_3$:CH_3OH (2:1). A 10 μL aliquot of each was spotted onto a HPTLC plate (Merck) and developed in a solvent system composed of $CHCl_3$:CH_3OH:H_2O (60:40:9) and 0.02% $CaCl_2$·$2H_2O$. The radioactive products were located by autoradiography. While GM_1 was the standard acceptor used in the above assays, additional acceptor substrates having terminal galactose residues were utilized as well in parallel assays. These were asialo-GM_1, nLc_4, Lc_4, lactosylceramide (Lc), and Gb_5, which were substituted for GM_1 at 30 μg each per assay reaction as described above.

Fucose Transfer to the GM_1 Acceptor in the Presence of a Variety of Detergents and Phospholipids. The effect of the lipidic environment on the activity of the expressed recombinant full-length rat hepatoma H35 cell α1→2FucT and the truncated rat H35 cell enzyme was assessed by assaying enzyme activity in the presence and absence of various detergents and phospholipids. The complete reaction mixture contained 2.5 μmol of HEPES buffer, pH 7.2, 30 μg of acceptor substrate, 100 μg of detergent or phospholipid, 1 μmol of $MnCl_2$, 0.5 mmol of ATP, 15 nmol of GDP-[^{14}C]-fucose (15 000 cpm/nmol), and enzyme in a total reaction volume of 0.1 mL. Detergents tested included CHAPSO, Triton X-100, Triton CF-54, deoxycholate (DOC), taurodeoxycholate (TDOC), and G-3634A. Phospholipids tested included phosphatidylinositol (PPI), dolichol phosphate, phosphatidylcholine (PPC), phosphatidylethanolamine (PPE), and phosphatidylglycerol (PPG).

Synthesis of Galactosyl Globoside (Gb_5) from Globoside (Gb_4). Globoside was purified from human erythrocytes as previously described (*27*). The enzymatic synthesis of Gb_5 was accomplished by the transfer of galactose from UDP-Gal to the acceptor substrate Gb_4 in a reaction catalyzed by β1→3GalT-V (*28*). The complete reaction mixture contained 5 mg of Gb_4, 5 mg of Triton X-100, 10 mg of UDP-Gal, 50 μmol of $MnCl_2$, 125 μmol of HEPES, pH 7.2, and enzyme in a total reaction volume of 5 mL. The reaction was incubated at 37 °C, and 5 μL aliquots were streaked onto an HPTLC plate at various times to monitor the glycosylation process. The HPTLC plate was washed extensively with water, dried, and developed in $CHCl_3$:CH_3OH:H_2O (50:40:10). Glycolipid bands were revealed by the orcinol/H_2SO_4 reaction. When the reaction was completed, the reaction mixture was passed over a reverse-phase C-18 column and eluted with methanol after extensive washing with water. The product was subjected to HPLC on an Iatrobeads 6RS 8010 column using a gradient elution of 2-propanol:hexane:H_2O from 55:40:5 to 55:25:20 over 200 min. Two milliliter fractions were collected and orcinol-positive fractions pooled according to HPTLC migration in $CHCl_3$:CH_3OH:H_2O (50:40:10). The pooled fraction of purified Gb_5 was dried under N_2 using a nitrogen evaporator and used as a precursor for the enzymatic synthesis of globo-H.

Preparative in Vitro Biosynthesis of (A) Fucosyl-GM_1 and (B) Globo-H Utilizing Recombinant Rat α1→2 Fucosyltransferase. (A) Preparative biosynthesis of fucosyl-GM_1 was conducted in reaction mixtures composed of 25 μmol of HEPES buffer, pH 7.2, 10 μmol of $MnCl_2$, 500 μg of CHAPSO, 0.5 mg of GM_1, and 2 mg of a crude cell homogenate of COS-7 cells transiently transfected with FL-RFT(+) plasmid in a total volume of 0.5 mL. The progress of the reaction was followed over time by withdrawing 2 μL of the reaction mixture and spotting it on an HPTLC plate. The plate was developed in a solvent system composed of $CHCl_3$:CH_3OH:H_2O (60:40:9) containing 0.02% $CaCl_2$. Glycolipid bands were determined by the orcinol/H_2SO_4 reaction to assess the completion of the glycosylation step. The reaction was stopped by dialyzing the product extensively with water and drying it down. The product was then purified by HPLC under the same conditions described for the purification of Gb_5. Approximately 0.5 mg of purified product was subjected to further structural analyses as described below.

(B) Fucosylgalactosyl globoside (globo-H) was enzymatically synthesized by transferring fucose from GDP-fucose to the acceptor substrate galactosyl globoside (Gb_5) in a reaction catalyzed by full-length expressed recombinant rat α1→2FucT. Gb_5 was biosynthesized as described above. The complete reaction mixture contained 2 mg of Gb_5, 2 mg of Triton CF-54, 4 mg of GDP-fucose, 20 μmol of $MnCl_2$, 50 μmol of HEPES, pH 7.2, and enzyme in a total reaction volume of 2 mL. The reaction was incubated at 37 °C, monitored by TLC as described above, and purified under the same conditions described for the purification of Gb_5. Approximately 1 mg of purified product was subjected to further structural analyses as described below.

^{1}H-Nuclear Magnetic Resonance Spectroscopy. A sample of biosynthetic fucosyl-GM_1 was prepared for NMR analysis by repeated lyophilization from D_2O (99.996 atom %; Cambridge Isotope Laboratories, Woburn, MA); a sample of Globo H was prepared by repeated addition and evaporation under a N_2 stream of methanol-d_4 (99.96 atom %; Cambridge Isotope Laboratories, Woburn, MA). Samples were then dissolved in 0.5 mL of DMSO-d_6 (99.96 atom %; Aldrich, Milwaukee, WI) containing 2% D_2O (*29*). All ^{1}H-NMR spectra were acquired on a Varian Inova 600 MHz Fourier transform spectrometer, at a probe temperature of 308 K, a sweep width of 5000 Hz, and with suppression of the residual HOD resonance by a presaturation pulse during the preparatory delay period. 1-D ^{1}H-NMR spectra were resolution enhanced prior to Fourier transformation. Anomeric and other "reporter" resonances were initially assigned by comparison with those published for the substrate GM_1 [complete assignments available, acquired at 30 °C (*30*)], Fuc-GM_1 isolated from PC12 rat pheochromocytoma cells [anomeric and Fuc H-5 assignments only, acquired at 27 °C (*31*)], and bovine thyroid (almost complete assignments, T. White, S. B. Levery, and H. Clausen, unpublished); globo-H isolated from a variety of sources [anomeric, Fuc H-5/H-6, and a few other tentative assignments available at various temperatures (*32*−*35*); and for other globo-series compounds (*29*, *32*, *36*−*38*) and type 1, 2, and 3 chain glycosphingolipids carrying the terminal histo-blood group H Fucα1→2Galβ1→3/4HexNAcβ1→ determinant (*39*−*41*)]. In addition, a series of 2-D ^{1}H-NMR experiments were performed which allowed

assignment of almost all nonexchangeable protons in the products. Acquisition and processing of 2-D $^1H-^1H$ PS-DQF-COSY (*42*, *43*), TOCSY (*44*, *45*), and PS-NOESY (*46*) experiments were performed and processed using standard Varian VNMR software.

RESULTS

Expression of the Ganglio-B Determinant and RT-PCR Analysis of α1→2FucT mRNA in Normal and AAF-Fed Rat Liver Tissues. Frozen sections from normal and AAF-fed rat liver were tested for expression of the B-determinant on a ganglio-series core chain using affinity-purified rabbit polyclonal antibody and FITC-labeled secondary goat anti-rabbit whole Ig. The results are shown in Figure 2. No antibody-positive regions of hepatocyte cell staining were detected in normal liver sections (panel A), while numerous discrete antibody positive foci were observed in rat liver sections after 5 weeks of AAF administration (panel B). Comparison of B determinant-positive foci with expression of γ-glutamyltranspeptidase (GGT)-positive foci (*47*) on doubly stained sections demonstrated mixed coexpression results. Focal staining showing both coincident and independently stained foci for each marker was observed (results not shown). Results of RT-PCR analysis of total RNA derived from H35 rat hepatoma cells and normal and AAF-fed rat liver tissues using primers specific for α1→2FucT are shown in Figure 2 (panel C). A single PCR product of approximately 0.6 kb was obtained from rat H35 hepatoma cells (lane 1) and from AAF-fed rat liver (lane 3), but not from normal rat liver (lane 2). All AAF-fed liver tissue samples used in this study were positive for α1→2FucT enzyme activity (results not shown).

Analysis of Expressed Recombinant Full-Length α1→2 Fucosyltransferase Activity. Expression of full-length α1→2FucT cDNA in transfected cells results in membrane-bound enzyme. As shown in Figure 3, the recombinant-expressed full-length enzyme transfers fucose to GM_1 with high efficiency. Comparable transfer was observed in the absence or presence of (100 μg) CHAPSO detergent (lanes 1 and 4, respectively). Somewhat less transfer to GM_1 was observed in the presence of 250 μg of phosphatidylglycerol (PPG) (lane 2), and significantly less transfer was observed in the presence of both PPG (250 μg) and G3634A detergent (100 μg) (lane 3). No fucose transfer was observed under any of these conditions in homogenates from COS-7 cells transfected with FL-RFT(−) (lanes 5−8).

Effect of Different Detergents and Phospholipids on the Activity of Recombinant Expressed Full-Length and Truncated Rat Hepatoma α1→2 Fucosyltransferase Enzymes. The effect of six different detergents on the activity of full-length recombinant α1→2FucT from FL-RFT(+) transfected COS-7 cell homogenates is shown in Table 1. The activity of full-length recombinant α1→2FucT was enhanced over 4-fold by Triton CF-54 as compared to the next best treatments, CHAPSO or no detergent. Equivalent activity was observed in the presence of CHAPSO detergent or with no exogenous detergent at 27% and 28%, respectively. Triton X-100 was mildly inhibitory at 23% while DOC, TDOC, and G3634A were severely inhibitory at 12%, 0%, and 16% optimal activity, respectively.

The effect of five different phospholipids on the activity of full-length recombinant α1→2FucT from FL-RFT(+) transfected COS-7 cell homogenates is shown in Table 2. Equivalent activity was observed in the presence of PPG or with no exogenous phospholipid at 23.1% and 22.3%, respectively. PPI, dolichol phosphate, and PPE were mildly inhibitory over no exogenous phospholipid at 14.8%, 12.1%, and 10.1%, respectively. PPC highly diminished α1→2FucT enzyme activity to 1.6% of optimal activity.

The effect of various detergents and phospholipids on the activity of truncated (CAT-RFT), IgG−Sepharose beaded α1→2FucT enzyme was tested as described above for the full-length enzyme. All conditions were tested in both the

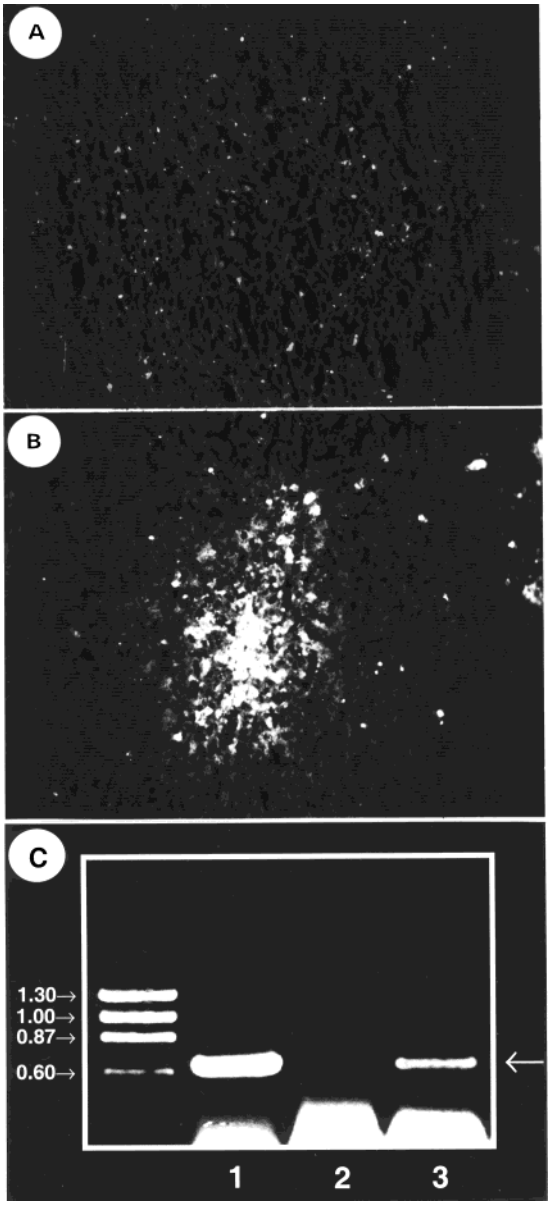

FIGURE 2: Screening of normal and AAF-fed rat liver tissues for expression of the ganglio-B determinant ($II^3NeuAcIV^3\alpha GalIV^2$-$FucGg_4$) and rat hepatoma α1→2FucT transcripts. Immunofluorescent staining of sections from (A) normal rat liver and (B) rat liver tissue after 5 weeks of 0.03% AAF feeding using affinity-purified rabbit polyclonal antibodies against $II^3NeuAcIV^3\alpha GalIV^2$-$FucGg_4$ and FITC-labeled goat anti-rabbit secondary Ig. (C) RT-PCR analysis of total RNA derived from (lane 1) rat hepatoma H35 cells, (lane 2) normal rat liver tissue, and (lane 3) AAF-fed rat liver tissue using primers specific for rat α1→2FucT. Seven microliters of each PCR mix was electrophoresed in a 0.8% agarose gel in 1× TBE buffer. The gel was stained with ethidium bromide. Size standards of 1.3, 1.0, 0.87, and 0.6 kb are indicated.

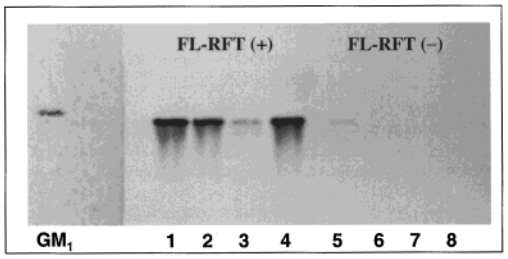

FIGURE 3: TLC analysis of reaction products from the transfer of [^{14}C]fucose to GM_1 catalyzed by expressed recombinant full-length rat hepatoma H35 cell α1→2 fucosyltransferase. Results from pcDNA3 expressed H35 cell α1→2FucT in the forward [FL-RFT-(+)] orientation (lanes 1−4) and reverse [FL-RFT(−)] orientation (lanes 5−8) are shown. Lanes: 1 and 5, transfer in the absence of exogenous detergent or phospholipid; 2 and 6, transfer in the presence of phosphatidylglycerol (PPG); 3 and 7, transfer in the presence of PPG and G3634A detergent; 4 and 8, transfer in the presence of CHAPSO detergent. TLC mobility of the GM_1 acceptor is indicated.

Table 1: Transfer of [^{14}C]Fucose to GM_1 Catalyzed by Expressed Recombinant Full-Length Rat α1→2 Fucosyltransferase in the Presence of Different Detergents

condition	fucose transfer (pM/h)	% optimal activity[a]
no detergent/acceptor	0	0
no detergent	101.3	28
CHAPSO	97.8	27
DOC	4.4	12
TDOC	0	0
Triton CF-54	364.2	100
G3634A	5.8	16
Triton X-100	83.5	23

[a] Values were normalized to Triton CF-54 detergent (treatment yielding the highest activity) at 100%.

Table 2: Transfer of [^{14}C]Fucose to GM_1 Catalyzed by Expressed Recombinant Full-Length Rat α1→2 Fucosyltransferase in the Presence of Different Phospholipids

condition	fucose transfer (pM/h)	% optimal activity[a]
Triton CF-54	537.7	100
no phospholipid	120.0	22.3
PPI	79.7	14.8
dolichol phosphate	64.8	12.1
PPG	124.4	23.1
PPE	54.4	10.1
PPC	8.6	1.6

[a] Values were normalized to Triton CF-54 detergent (treatment yielding the highest activity) at 100%.

presence and absence of exogenous membrane homogenates from untransfected COS-7 cells. These homogenates were added in some cases as a membrane control for any properties inherent in COS-7 cell membranes which might influence activity and behavior of the beaded truncated enzyme. No endogenous α1→2FucT enzyme activity was detected in untransfected COS-7 cell homogenates tested in the presence of GM_1 acceptor and Triton CF-54 or CHAPSO detergent. No CAT-RFT catalyzed fucose transfer to GM_1 was detected in the absence of exogenous detergent or phospholipid with or without added COS-7 cell membranes. Of all the conditions tested, only CHAPSO detergent significantly activated the truncated enzyme (results not shown). The presence of untransfected COS-7 cell membrane homogenates in assay cocktails had no effect on CAT-RFT activity in most cases, but strongly inhibited the activation of CAT-RFT enzyme by CHAPSO detergent diminishing CAT-RFT activity to 38% of that observed in the presence of CHAPSO alone (results not shown).

Table 3: Transfer of [^{14}C]Fucose to GM_1, Asialo-GM_1 (Gg_4), and Galactosyl Globoside (Gb_5) Acceptors Catalyzed by Native, Expressed Recombinant Full-Length, and Truncated Rat α1→2 Fucosyltransferase

		fucose transfer (pM/h)		
acceptor	condition	native	FL-RFT	CAT-RFT
GM_1	(+) detergent[a]	8.0	32.4	11.3
	(−) detergent	6.2	6.0	0.0
Gg_4	(+) detergent	8.4	95.7	4.6
	(−) detergent	0.7	1.1	0.0
Gb_5	(+) detergent	4.0	24.2	0.9
	(−) detergent	0.4	5.4	0.0

[a] Triton CF-54 detergent was used in H35 native and expressed recombinant full-length enzyme assays. CHAPSO detergent was used in truncated enzyme assays.

Determination of the Specific Activity of Recombinant Expressed Full-Length and Truncated Rat Hepatoma α1→2 Fucosyltransferase Enzymes. To allow direct comparison of the specific activities of the membrane-bound full-length and soluble truncated enzyme forms, both constructs were expressed in a vector (pcDNA3.1/NT-GFP-TOPO) resulting in expression of GFP fusion proteins for ease of protein quantitation. On the basis of α1→2FucT enzyme assays (GM_1 acceptor/standard conditions) of homogenates of membranes from COS-7 cells transfected with GFP/FL-RFT or GFP/CAT-RFT and quantitation of cycle 3 GFP/α1→2FucT fusion proteins via western blotting, the specific activities of the expressed recombinant full-length and truncated α1→2FucT enzymes were determined to be 535.8 and 6.58 nmol/h/mg protein, respectively. Other studies indicate that the behavior and activities of the GFP:α1→2FucT fusion protein enzymes are essentially identical to FL-RFT and CAT-RFT enzymes (results not shown).

Substrate Specificity of Native, Expressed Recombinant, and Truncated Rat Hepatoma α1→2 Fucosyltransferase Enzymes. The results of a study in which different acceptors bearing terminal Galβ1→3GalNAc- structures were fucosylated by native H35, truncated, and full-length α1→2FucT enzymes are shown in Table 3. Assays were conducted using quantities of CAT-RFT beaded enzyme, H35 cell homogenate, or FL-RFT(+) transfected COS-7 cell homogenate, yielding equivalent amounts of activity on GM_1 acceptor. All three enzyme forms were observed to transfer fucose to GM_1, Gg_4, and Gb_5 acceptors, but only the native and full-length recombinant enzymes fucosylated these acceptors in the absence of detergent. Fucose transfer to lacto- and neolacto-series acceptors catalyzed by the full-length [FL-RFT(+)] and truncated (CAT-RFT) enzyme forms was examined in a parallel study using Lc, Lc_4, and nLc_4 as well as GM_1, Gg_4, and Gb_5 acceptor substrates. Only expressed recombinant full-length α1→2FucT provided detectable product with Lc, Lc_4, and nLc_4 acceptors. Relative fucose transfer to all six acceptors is shown in Table 4.

Structural Determination of Reaction Products from Fucosylation of GM_1 and Gb_5 Acceptors by Rat Hepatoma α1→2 Fucosyltransferase. Scaled-up reaction mixtures for the preparative biosynthesis of fucosyl-GM_1 and Globo H

Table 4: Fucose Transfer to Various Glycosphingolipid Acceptors Catalyzed by Expressed Recombinant Full Length and Truncated α1→2Fucosyltransferase Enzymes

acceptor	FL-RFT[a]	CAT-RFT
GM_1	1.0	1.0
Gg_4	1.98 ± 0.51	0.53 ± 0.13
Gb_5	0.67 ± 0.05	0.04 ± 0.02
Lc	0.04 ± 0.01	ND
Lc_4	0.16 ± 0.07	ND
nLc_4	0.07 ± 0.04	ND

[a] Mean ± standard error. Relative fucose transfer normalized to the activity found with GM_1 as the acceptor is shown. ND = not detectable or within 1 standard deviation of zero.

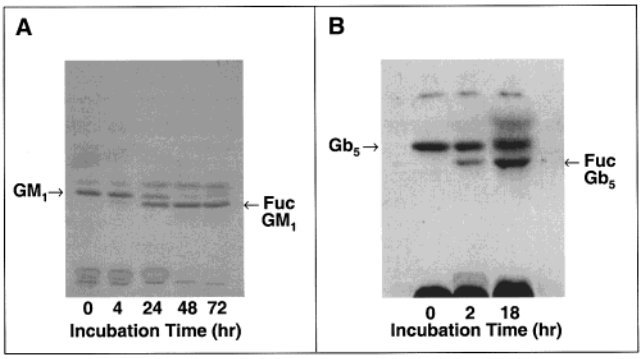

FIGURE 4: Preparative in vitro biosynthesis of fucosyl-GM_1 and globo-H utilizing expressed recombinant full-length rat α1→2 fucosyltransferase. (A) The appearance of increasing amounts of a slower migrating band, corresponding to fucosyl-GM_1 from transfer of fucose in the α1→2 linkage to the added GM_1 acceptor, is shown over time. TLC mobilities of GM_1 and fucosyl-GM_1 are indicated. (B) The appearance of increasing amounts of a slower migrating band, corresponding to globo-H from transfer of fucose in the α1→2 linkage to the added galactosyl globoside (Gb_5) acceptor, is shown over time. TLC mobilities of Gb_5 and fucosyl-Gb_5 are indicated.

were set up to generate sufficient glycosphingolipid for subsequent structural determinations by ^{1}H-NMR spectral analysis. Biosynthesis of the reaction products were followed over time (Figure 4). Panel A shows conversion of GM_1 to fucosyl-GM_1 catalyzed by FL-RFT over a 72 h time period. Over 50% conversion of GM_1 acceptor to fucosyl-GM_1 was observed within 24 h. By 48 h, the entire starting amount (0.5 mg) of GM_1 acceptor substrate was converted to fucosyl-GM_1. Panel B shows biosynthesis of fucosylgalactosyl globoside (globo-H structure) catalyzed by FL-RFT over 18 h. Detectable levels of globo-H were observed within 2 h, and about 50% of the starting amount (2.0 mg) of the galactosyl globoside acceptor substrate was converted to globo-H by the end of the 18 h incubation period. The enzymatic pathways used to synthesize fucosyl-GM_1 and globo-H are shown in Figure 5.

^{1}H-Nuclear Magnetic Resonance Spectroscopic Analysis of Fucosyl-GM_1 and Globo-H. The biosynthetic glycosphingolipid products were subjected to analysis by ^{1}H-NMR spectroscopy (see Figure 6) in order to verify that FL-RFT catalyzed incorporation of fucose in the proper linkage position and configuration. Due to the limited published data available for the putative products, the assignments were verified by a series of 2-D ^{1}H-NMR experiments which allowed assignment of almost all nonexchangeable protons in the products (listed in Tables 5 and 6).

An expansion of the downfield region of a 1-D ^{1}H-NMR spectrum of the fucosyl-GM_1 product is reproduced in Figure 6A. Compared with that of GM_1, the spectrum is readily characterized by the presence of an α-anomeric resonance ($^3J_{1,2}$ = 2−4 Hz) at 5.138 ppm ($^3J_{1,2}$ = 4.0 Hz) in addition to the four β-anomeric resonances ($^3J_{1,2}$ = 7−9 Hz) contributed by the substrate glycan. Observation of a characteristic H-5 quartet at 4.044 ppm, coupled to an upfield H-6 (CH_3) doublet at 1.060 ppm, is further evidence that this corresponds to an α-Fuc residue. Interestingly, the chemical shift of the α-Fuc H-1 resonance varies considerably from those found in all other glycosphingolipids having a type 1, 2, or 3 chain terminal H-trisaccharide determinant Fucα1→2Galβ1→3/4HexNAcβ1→ (*32*−*35*, *39*, *41*), undoubtedly due to the influence of the branching NeuAc residue. On the other hand, the glycosylation-induced chemical shift changes observed for β-Gal IV H-1 through H-4 (Δδ = +0.23, +0.16, +0.28, +0.02, respectively) and for β-GalNAc III H-1 through H-3 (Δδ = −0.16, +0.05, +0.23, respectively) are very similar in both sign and relative magnitude to those analogous changes which can be observed for β-Gal IV and β-GlcNAc III H-1 of type 1 chain Lc_4Cer upon addition of Fucα1→2 to form the corresponding type 1 chain H glycosphingolipid (*39*). Unfortunately, due to the exact chemical shift coincidence of β-Gal IV H-2 with H-2 of the α-Fuc residue, ready confirmation of the Fucα1→2Gal linkage by observation of an interglycosidic nuclear Overhauser enhancement was precluded by the strong intraresidue nOe observed as expected between H-1 and H-2 of the α-Fuc. However, since no other nOe was observed, and since the ^{1}H NMR spectral data for the biosynthetic Fuc-GM_1 were essentially the same as those from the natural compound isolated from bovine thyroid (T. White, S. B. Levery, and H. Clausen, unpublished) in both one and two dimensions, it appears established that the compounds are identical with respect to glycan structure and the terminal α1→2 linkage of the Fuc residue in the biosynthetic product therefore confirmed.

The 1-D ^{1}H-NMR spectrum of the biosynthetic globo-H product (Figure 6B) was virtually identical to those obtained previously from V^2FucGb_5Cer originally isolated from the human teratocarcinoma cell line 2102Ep (*32*), from normal and neoplastic human mammary tissue (*32*), and from human histo-blood group H erythrocytes (*34*) and kidney tissue (*35*), particularly to those acquired under identical conditions (*33*, *34*). In this case, the sugar residues of the core Gb_5 glycan are represented by five anomeric resonances, four β- and one α-, as expected, while the additional α-Fuc residue is represented by reporter resonances for H-1, H-5, and H-6 at 4.959 ($^3J_{1,2}$ = 2.9 Hz), 4.077, and 1.088 ppm. 2-D ^{1}H-NMR experiments allowed confirmation of the assignment of the broadened triplet at 4.102 ppm to H-5 of α-Gal III, as well as a number of other assignments made previously based on analogy (*32*, *34*). As with Fuc-GM_1, the glycosylation-induced shift changes for H-1 of β-Gal V and β-GalNAc IV (Δδ = +0.27, −0.13, respectively, compared to those resonances in the substrate Gb_5Cer) are also consistent with addition of Fucα1→2 to the terminal β-Gal of a Galβ1→3HexNAcβ1→ disaccharide (*32*, *39*).

DISCUSSION

The results indicate that the activation of α1→2FucT in rat liver following AAF administration, resulting in the synthesis of ganglio-B determinant containing structures,

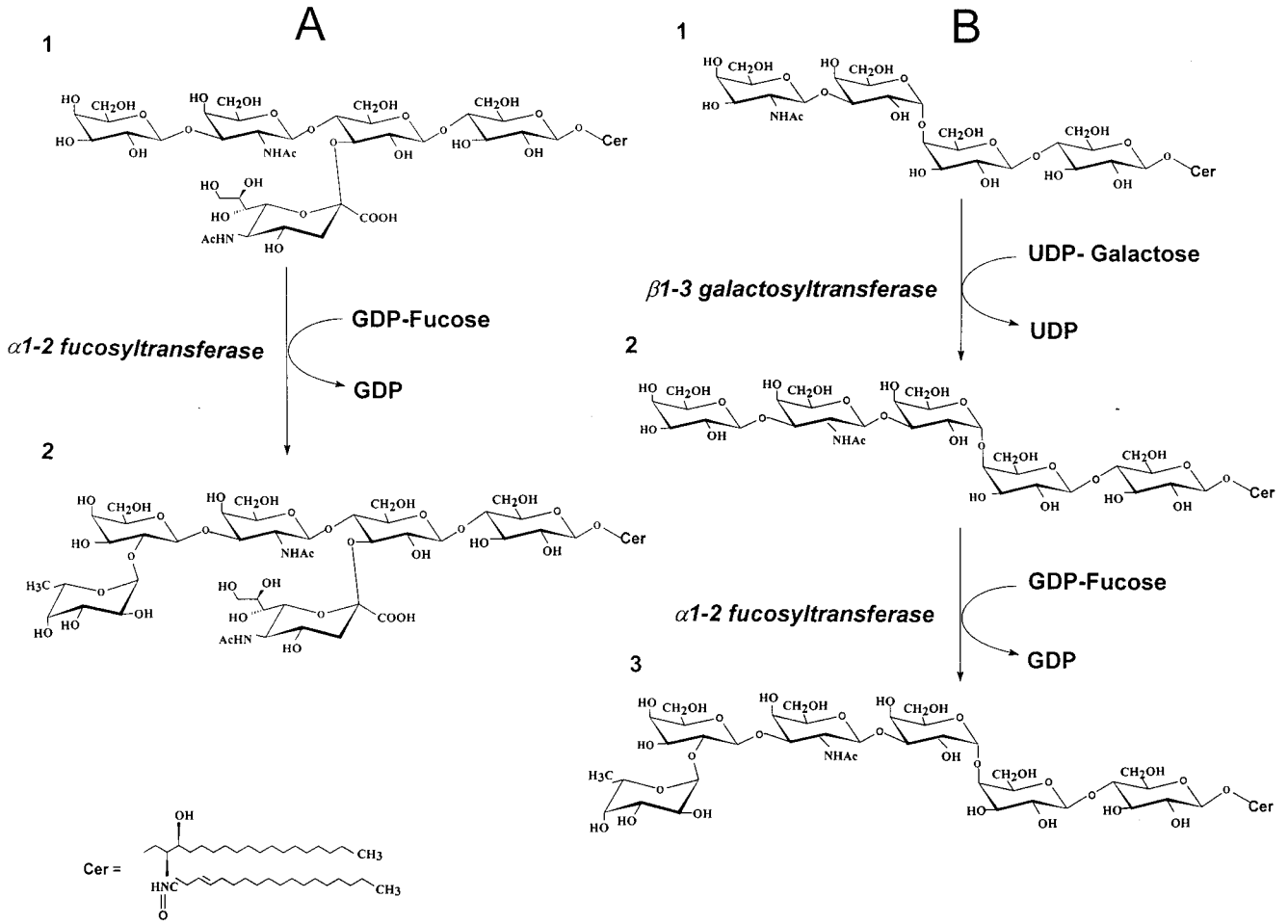

FIGURE 5: Enzymatic pathways used to synthesize fucosyl-GM_1 and globo-H. (A) Biosynthesis of fucosyl-GM_1 from GM_1: 1, GM_1 ($II^3NeuAcGg_4$, Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glcβ1→1Cer); 2, fucosyl-GM_1 ($II^3NeuAcIV^2FucGg_4$, Fucα1→2Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glcβ1→1Cer). (B) Two-step biosynthesis of globo-H (fucosylgalactosyl globoside) from globoside (Gb_4): 1, (Gb_4) globoside (GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer); 2, (Gb_5) galactosyl globoside (Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer); 3, (V^2FucGb_5) globo-H (Fucα1→2Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer).

occurs in association with the series of events leading to hepatocarcinogenesis (*3*, *4*, *6*). It has been postulated that expression of such structures on tumor cells might provide a means for tumor cells to escape recognition by host cellular immunity (*2*, *3*). Recent studies have demonstrated that elevated cellular ganglioside expression increases the metastatic potential of transformed cells (*48*), and it has been suggested that shedding of immunosuppressive gangliosides may be a mechanism used by neoplastic cells to inhibit host immune responses and thus escape detection by the immune system (*48*−*51*). Thus, by the same line of reasoning, expression of these fucogangliosides early in the transformation process may increase the survival advantage of potentially initiated hepatocytes, thereby enhancing the probability that they will undergo malignant transformation (*2*). In the present study, a striking correlation has been observed between induction of α1→2FucT activity, expression of ganglio-B structures as determined by binding of highly specific antibody to hepatocytes, and the appearance of RT-PCR detectable transcripts for the rat hepatoma GDP-fucose: GM_1 α1→2FucT in AAF-fed rat liver tissues. Given that these events are closely associated with early stage processes of transformation in rat liver which often persist in derived hepatomas (*1*, *2*, *5*, *6*, *17*), it is possible that this induction occurs in hepatocyte cell subpopulations more apt to be enriched in initiated cells.

Mammalian membrane-bound glycosyltransferases are composed of a short cytoplasmic N-terminal domain, a transmembrane domain, a luminal stem region, and a C-terminal catalytic domain. In most cases, the cytoplasmic N-terminal domain, the transmembrane domain, and portions of the stem region can be removed and are not required for catalytic activity. For example, in previous studies utilizing human α1→3FucT enzymes, few differences in activity or acceptor specificity properties were seen between full-length and truncated recombinant chimeric (protein A fusion) enzyme forms (*23*, *24*, *52*). Further, studies with a variety of glycosyltransferases expressed as a soluble catalytic domain in a baculovirus system (*53*−*55*) demonstrated high enzymatic activity when the N-terminal transmembrane domain was deleted. In contrast, this does not appear to be the case in the comparison of the rat hepatoma α1→2FucT full-length and truncated enzyme forms. The full-length recombinant enzyme has a specific activity (535.8 nmol/h/mg protein) over 80-fold higher than that of the truncated enzyme (6.58 nmol/h/mg protein) and does not require exogenous detergent or phospholipid for activation. Most detergents mildly to severely inhibit α1→2fucosyltransferase activity in FL-RFT(+) transfected COS-7 cell homogenates. The only detergent found to significantly enhance enzyme activity was Triton CF-54. CHAPSO detergent neither inhibits nor enhances activity of the full-length enzyme but

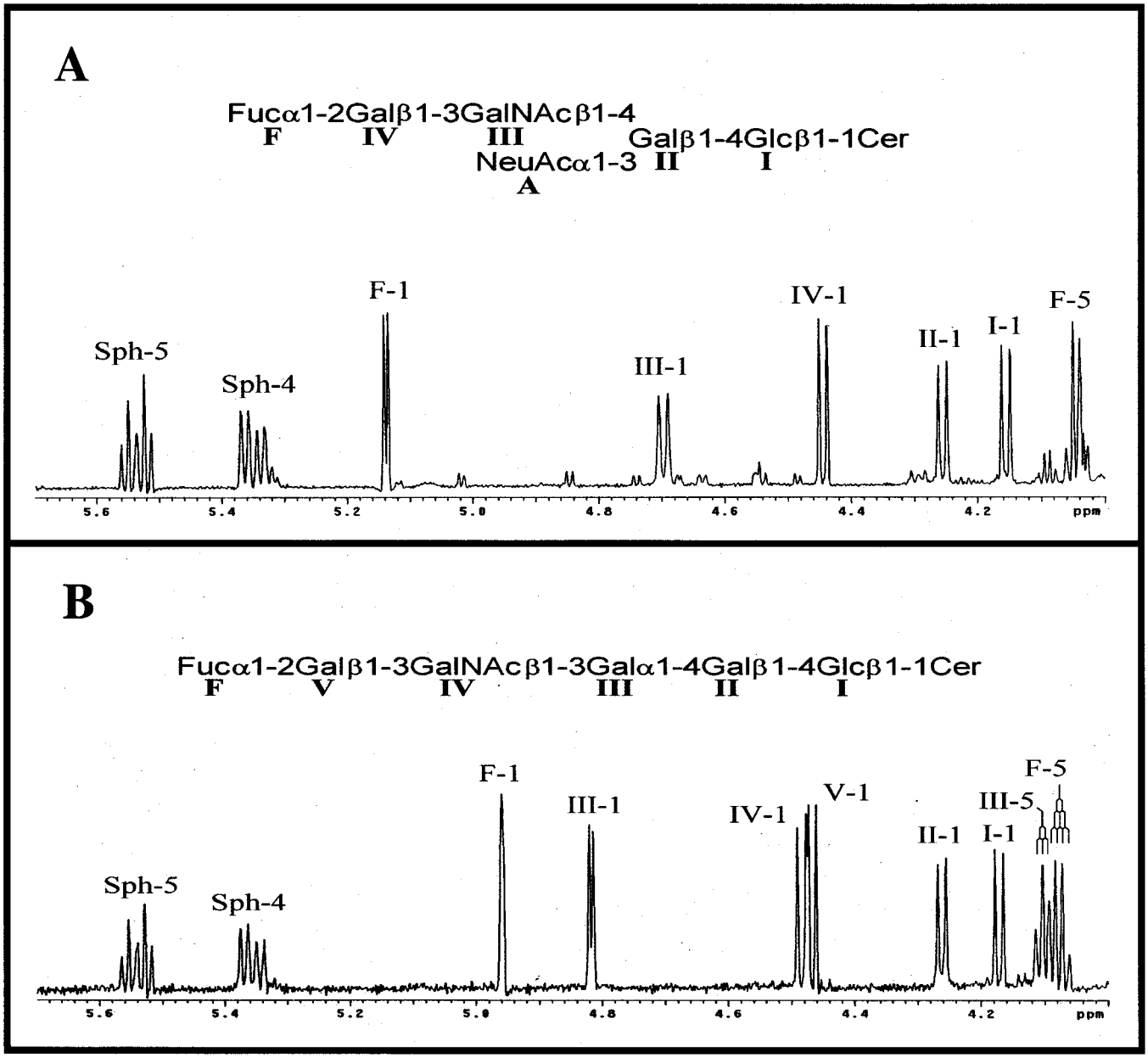

FIGURE 6: Downfield regions of 1-D ^{1}H-NMR spectra of biosynthetic fucosyl-GM_1 (panel A) and globo-H (panel B) in DMSO-d_6/D_2O at 308 K. Arabic numerals refer to ring protons of residues designated by Roman numerals or capital letters in the corresponding structure drawn at the top of the figure. Sph refers to protons of the sphingosine backbone.

was the only detergent tested which significantly activated the truncated form. These results are similar to those for the native enzyme, as previous enzymological studies indicated that the native enzyme was inhibited by a wide variety of detergents but was stable in the presence of 0.4% CHAPSO detergent (*19*). The truncated enzyme lacks the 27 amino acid transmembrane/cytoplasmic domain and requires CHAPSO for optimal activity, presumably to afford more efficient interaction of the lipidic acceptor with the enzyme active site.

It has been proposed that membrane phospholipids may play a role in maintaining optimal activity of the native enzyme (*19*). The activity of the recombinant full-length rat hepatoma α1→2FucT enzyme in the presence of a number of phospholipids was tested. Most of these had an inhibitory effect on recombinant expressed full-length enzyme activity. PPG was neutral and PPC was almost completely inhibitory. It has previously been reported that the overall activity but not binding affinity of the H35 cell native enzyme is enhanced by PPG and the cationic detergent G3634A (*18*, *19*). However, in the present study, the activity of the full-length recombinant enzyme as expressed in COS-7 cells was found to be significantly inhibited by this combination. The truncated enzyme was not appreciably activated by any phospholipids tested in either the presence or absence of homogenized COS-7 cell membranes. Untransfected COS-7 cell membrane homogenates were added in some cases to CAT-RFT assay cocktails in order to mimic the lysate conditions of the full-length enzyme but were found to have little effect on net CAT-RFT activity in general. Thus, lipidic environment clearly appears to play a different role in modulating the activity of the native and full-length enzymes as compared to the truncated form, which is missing the transmembrane portion of the enzyme.

Table 5: Proton Chemical Shifts (ppm from Tetramethylsilane) and $^3J_{1,2}$ Coupling Constants (Hz) for Biosynthetic Ganglio-H Antigen in Dimethyl Sulfoxide-d_6/2% D_2O at 308 K

	Fucα1→2Galβ1→3GalNAcβ1→4 (NeuAcα2→3) Galβ1→4Glcβ1→1Cer						
	F	IV	III	A	II	I	R
H-1	5.138	4.444	4.697		4.254	4.155	3.420 (a) 3.980 (b)
($^3J_{1,2}$)	(4.0)	(7.5)	(8.6)		(8.0)	(7.8)	
H-2	3.529	3.529	3.958		3.183	3.040	3.776
H-3	3.529	3.586	3.705	2.550 (eq) 1.642 (ax)	3.742	3.334	3.883
H-4	3.685	3.633	3.802	3.705	3.888	3.284	5.351
H-5	4.044	3.407	3.644	3.381	3.451	3.284	5.536
H-6	1.060 (3H)	ND	ND	3.178	3.482 (a) 3.642 (b)	3.620 (a) 3.745 (b)	ND
H-7				3.190			
H-8				3.500			
H-9				3.345 (a) 3.620 (b)			
NAc			1.753	1.875			

Table 6: Proton Chemical Shifts (ppm from Tetramethylsilane) and $^3J_{1,2}$ Coupling Constants (Hz) for Biosynthetic Globo-H Antigen in Dimethyl Sulfoxide-d_6/2% D_2O at 308 K

	Fucα1→2Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer						
	F	V	IV	III	II	I	R
H-1	4.959	4.467	4.483	4.817	4.262	4.171	3.449 (a) 3.962 (b)
($^3J_{1,2}$)	(2.9)	(7.3)	(8.3)	(3.7)	(7.9)	(7.0)	
H-2	3.511	3.515	3.885	3.755	3.292	3.041	3.787
H-3	3.511	3.496	3.730	3.594	3.399	3.291	3.874
H-4	3.489	3.646	3.503	3.963	3.814	↓[a]	5.356
H-5	4.077	ND	3.406	4.102	3.568	3.291	5.541
H-6	1.088 (3H)	ND	ND	3.459 (a) 3.490 (b)	3.675 (a) 3.584 (b)	3.618 (a) 3.748 (b)	1.936
NAc			1.823				

[a] Strongly coupled H-3, H-4, and H-5, centered at 3.291 ppm.

Previous experiments with H35 cell extracts demonstrated that transfer to neolacto-series acceptors occurred at a rate only 2% of that found for GM_1 (*18*). Results of the present study confirmed that fucose is transferred weakly to nLc_4 (and Lc and Lc_4 acceptors as well) by the recombinant expressed full-length α1→2FucT at rates of 7.0%, 4.0%, and 16%, respectively, of that found for GM_1. The truncated enzyme did not transfer fucose to any of these acceptors at detectable levels, presumably due to its low inherent activity. Both the full-length and truncated enzymes catalyze the transfer of fucose to GM_1, asialo-GM_1, and galactosyl globoside but with different efficiencies. The full-length enzyme fucosylates asialo-GM_1 twice as efficiently as it fucosylates GM_1 as determined by relative fucose transfer, while the truncated form fucosylates this acceptor only half as efficiently as GM_1. Fucose is transferred to galactosyl globoside at 67% the level of transfer to GM_1 by the full-length enzyme, while the truncated enzyme only weakly transfers fucose to this acceptor at 4% as compared to GM_1. Despite these differences, the present study clearly demonstrates that the rat hepatoma H35 cell α1→2FucT has a strong preference for acceptor substrates bearing terminal Galβ1→3GalNAc- structures. This specificity is quite restricted as compared to other cloned α1→2fucosyltransferases but is very similar to that reported recently for three distinct bovine α1→2FucT genes (*13*) which are homologous to human *FUT1*, *FUT2*, and *Sec1*. The cloned rat hepatoma H35 cell α1→2FucT has an acceptor specificity most closely resembling that of secretor-type enzymes (*7*, *8*, *11*−*13*, *15*) and bears the greatest homology to the human *Sec2* gene (*17*).

The rat hepatoma H35 α1→2FucT efficiently transfers fucose to GM_1, asialo-GM_1, and galactosyl globoside acceptors, giving rise to a number of known tumor-associated antigens. For example, fucosyl-GM_1 has been identified as a selective tumor-associated marker of small cell lung

carcinoma (SCLC) cells (*56*) and has been detected as an important serum marker in SCLC patients (*57*). Enhanced expression of the carbohydrate structure, globo-H or fucosylgalactosyl globoside, is widely reported in many human carcinomas. It is abundantly expressed in human embryonal (*32*) and breast carcinomas (*33*, *58*) and in colon, lung, ovary, and small cell lung carcinomas (*59*) as well as in the majority of the carcinomas of pancreas, stomach, prostate, and uterine endometrium (*60*). It is also found in normal tissues such as fetal and adult epithelia of the kidney and ureter (*35*, *61*) and erythrocytes of A2 and O individuals (*62*). Globo-H is a normal component in kidney tissues of secretors (those individuals who express the α1→2FucT *Sec2* gene for conversion of galactosyl globoside to the terminal globo-H structure), while nonsecretors have been found to express sialylated galactosyl globoside or SSEA4, a receptor for uropathogenic *Escherichia coli* (*38*). We have confirmed that the recombinant expressed full-length α1→2FucT enzyme form is capable of rapidly generating two important cancer-associated antigens, fucosyl-GM_1 and globo-H. Thus, this enzyme may be particularly useful for in vitro preparation of large quantities of these moieties, for example, in potential cancer vaccine development.

In conclusion, we have cloned and expressed a highly active recombinant form of the GDP-fucose:GM_1 α1→2FucT enzyme associated with hepatocarcinogenesis in rat liver, have described a number of its properties, and have demonstrated the ability of this enzyme to efficiently transfer fucose to specific glycosphingolipid acceptors to produce important cancer-associated antigens. This enzyme represents a highly effective tool in generating some of these fucosylated structures for future investigations into the process of carcinogenesis and disease pathology.

ACKNOWLEDGMENT

We thank Bill Davis for fine technical assistance and gratefully acknowledge Dr. Henrik Clausen, D.D.S., of the Faculty of Health Sciences, School of Dentistry, Copenhagen, Denmark, for contribution of the cloned β1→3GalT-V enzyme used in the synthesis of galactosyl globoside (Gb_5) from globoside (Gb_4).

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BI0102104