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Fucosylation of Complex Glycosphingolipids by Recombinant Fucosyltransferase-VII[1]

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Fucosyltransferase VII (FucT-VII) is one of five known α1→3fucosyltransferases capable of transferring fucose to the C-3 position of *N*-acetylglucosamine residues found in lactosamine based glycans. Previous studies have indicated that FucT-VII has a very restricted specificity, capable of fucosylating only terminally α2→3sialylated carbohydrate substrates, resulting in the synthesis of the sialyl Lewis $\times$ (sLe^x) epitope. Although FucT-VII is expressed in cells of myeloid origin, the monosialylganglioside fraction of HL60 cells contains only internally and/or multiply fucosylated polylactosamine structures; no monofucosylated sLe^x derivatives are detected. We now report that the structure of the final product formed by the action of FucT-VII on sialy*nor*hexaosylceramide (a glycosphingolipid substrate having multiple fucosylation sites) is extended monofucosyl sLe^x and fucosylation is restricted to the terminal GlcNAc-V. This indicates that the biosynthesis of all fucosylated monosialylated gangliosides found in HL60 cells (including the E-selectin binding fractions) involves at least one additional α1→3fucosyltransferase.

Recent evidence suggests that α1→3fucosylation of sialylated lactosamine based glycoconjugates is the final step in the biosynthesis of the ligands recognized by the selectin family of cell adhesion molecules (**1**). The N-terminal region of E- and P-selectin contains a carbohydrate binding domain believed to recognize some sialylated and/or fucosylated glycoconjugate expressed on the surface of leukocytes. Endothelial expression and subsequent binding of E- and P-selectin to these glycans initiates the recruitment of leukocytes to sites of inflammatory lesions following infection and wounding (**2**).

To date, five α1→3fucosyltransferases (FucT-III, FucT-IV, FucT-V, FucT-VI, and FucT-VII) have been identified and cloned by various molecular biological techniques (**1**). With the exception of FucT-IV, all are capable of efficiently synthesizing the E-selectin ligand sialyl Lewis $\times$ (sLe^x) (**3-7;** see Table 1 for structures referred to in this study). Of the known fucosyltransferases only FucT-IV and FucT-VII are expressed in cells of myeloid origin, hence, one or both of these enzymes is responsible for synthesizing the true physiological ligand for E-selectin. Although much evidence has accumulated suggesting FucT-VII as a key enzyme (**8**), structural studies involving the monosialylgangliosides of HL60 cells indicate the absence of any monofucosylated sLe^x structures (**9,10;** Table II). Since all of the E-selectin binding fractions from HL60 cell gangliosides consisted of multiply fucosylated polylactosamine structures and the products of FucT-VII have not been thoroughly analyzed with respect to more complex structures, we characterized the final biosynthetic product generated by a recombinant FucT-VII on a sialylated lactosamine based substrate (sialyl*nor*hexaosylceramide) containing multiple fucosylation sites. The use of sialyl*nor*hexaosylceramide as a substrate for the *in vitro* studies presented here reflects more accurately the makeup of the endogenous substrates that FucT-VII acts on in HL60 cells.

MATERIALS AND METHODS

Glycolipid preparation. All glycolipid samples used in this study were either isolated or enzymatically synthesized in this laboratory (see Table I for structures of glycolipids referred to in this paper). VI^{3}NeuAcnLc$_4$ and VI^{3}NeuAcnLc$_6$ were purified from bovine erythro-

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Abbreviations: Cer, ceramide; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; HPTLC, high performance thin-layer chromatography; NeuAc, *N*-acetylneuraminic acid. Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Lipids* (1977) **12,** 455–463]; however, the suffix -OseCer is omitted.

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TABLE I

Structures of Glycosphingolipids Referred to in This Study

Glycosphingolipid	Structure
$IV^3NeuAcnLc_4$ (sPG)	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
$IV^3NeuAcIII^3FucnLc_4$ (sLe^x)	NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
nLc_6	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
$V^3FucnLc_6$	Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
$III^3FucnLc_6$	Galβ1-4GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
$VI^3NeuAcnLc_6$	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
$VI^3NeuAcV^3FucnLc_6$ (sY^2)	NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
$VI^3NeuAcIII^3FucnLc_6$ (VIM2)	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glcβ1-1Cer

cytes as previously described (**11**). Lacto*nor*hexaosylceramide (nLc_6) was prepared by desialylation of $VI^3NeuAcnLc_6$. Desialylation was performed in 1% acetic acid at 100°C for 1hr. ^{14}C-labeled glycolipid standards $III^3FucnLc_6$ and $V^3FucnLc_6$ were prepared as described below using HL60 cells as the enzyme source and both taurodeoxycholate and G-3634-A as detergents (**12**).

Transfection and expression of recombinant FucT-VII. Full length FucT-VII was constructed and expressed in pCDM8 as previously described (**7**). COS-7 cells were transfected with pCDM8 FucT-VII constructs using the DEAE dextran technique (**13**). COS-7 cells transfected with pCDM8 FucT-VII were harvested three to five days post transfection and stored frozen at −80°C before use.

Enzyme preparation. FucT-VII, FucT-IV, and FucT-III were isolated from COS-7 cells transfected with pCDM8 FucT-VII constructs, HL60 cells, and Colo 205 cells respectively as follows. Cells, 0.5mL, were thawed and homogenized in 2 volumes of 50 mM Hepes buffer, pH 7.2, 25% glycerol, 1mM EDTA, 0.2% TritonX-100R by two strokes of a Potter-Elvehjem homogenizer and used as the enzyme source in these studies. The enzymes were stored at −20°C until used in the following experiments.

Biosynthesis of fucosylated glycolipids. Unless otherwise specified, all reactions were carried out in mixtures containing 2.5 μmol Hepes buffer, pH 7.2, 30 μg acceptor glycolipid, 100 μg taurodeoxycholate, 1 μmol $MnCl_2$, 15 nmol GDP[^{14}C]fucose (15,000 cpm/nmol), and 15-150 μg protein in a total reaction volume of 0.1mL (**14**). The reaction mixture was incubated for 2 hours at 37°C and stopped by the addition of 100 μL $CHCl_3$:CH_3OH (2:1). The entire reaction mixture was streaked onto a 4-cm wide strip of Whatman 3 paper and chromatographed with water overnight. The glycolipid remaining at the origin was extracted twice with 5-mL washes of $CHCl_3$:CH_3OH:H_2O (10:5:1). The solvent was removed with a nitrogen stream and dissolved in 20μL $CHCl_3$:CH_3OH (2:1). An aliquot, 10 μL, was removed and spotted onto an HPTLC plate (Silica gel 60; EM Science, Gibbstown, NJ) and developed in a solvent system consisting of $CHCl_3$:CH_3OH:H_2O (50:40:10) containing 0.05% $CaCl_2$ as a final concentration.

The labeled product formed when $VI^3NeuAcnLc_6$ was used as a substrate in the above reaction with FucT-VII was located by autoradiography, scraped from the plate and extracted from the silica by sonication in $CHCl_3$:CH_3OH:H_2O (10:5:1). The silica was removed by centrifugation, re-extracted, and the combined solvent fractions were dried under N_2. The sample was desialylated by heating at 100°C in 1% acetic acid for 1 hr and cleaned up using a 1 cm^3 C-18 Sep-Pak cartridge (Waters, Milford, MA) with elution by absolute methanol. The desialylated radiolabeled product was developed in n-propanol:H_2O:NH_4OH (7:3:1), and located by autoradiography.

RESULTS

Fucosylation of sialyl*para*globoside and sialyl*nor*-hexaosylceramide ($snLc_6$) using recombinant FucT-VII and ^{14}C-labeled GDP-fucose resulted in the generation of a single monofucosylated product (Fig. 1A) for both substrates when observed by HPTLC in $CHCl_3$:CH_3OH:H_2O (50:40:10). Notably, there was no evidence of any difucosylated derivatives formed from $snLc_6$ using FucT-VII as seen when FucT-III from Colo 205 cells was used as the enzyme source (Fig. 1A). No transfer of fucose was seen when the neutral glycolipids nLc_4 or nLc_6 were used as acceptors (data not shown). The lack of an ability of FucT-VII to catalyze synthesis of difucosyl structures is confirmed by the data shown in Fig. 1B. Although the enzyme found in crude Colo 205 cells (primarily FucT-III) efficiently converted ^{14}C-

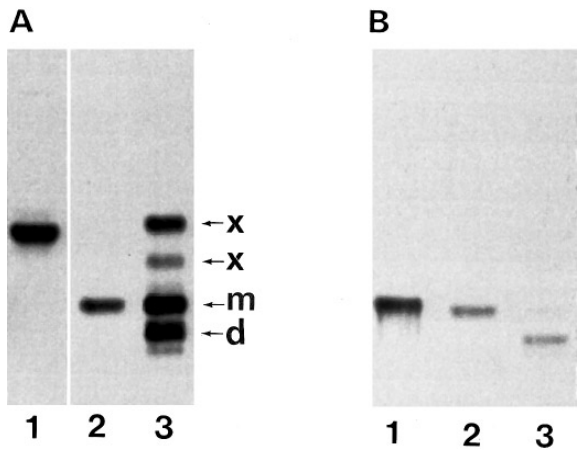

FIG. 1. Thin-layer chromatographic analysis of reactions products from $snLc_4$, $snLc_6$, and sY^2 catalyzed by recombinant FucT-VII. The reaction products resulted from the transfer of [^{14}C]fucose as described in Materials and Methods. Plates were developed in $CHCL_3$:CH_3OH:H_2O, 50:40:10. (A) Lane 1, autoradiograph of product when standard $snLc_4$ was used as an acceptor; lane 2, autoradiograph of product when standard $snLc_6$ was used as acceptor, lane 3, autoradiograph of products from $snLc_6$ catalyzed by Colo 205 α1→3fucosyltransferase as a control. Bands marked "x" resulted from transfer to acceptors endogenous to the crude Colo 205 enzyme fraction; m - monofucosyl product, d - difucosyl product. (B) Lane 1, autoradiograph of [^{14}C]-labeled sY^2 standard; lane 2, autoradiograph of [^{14}C]-labeled sY^2 reacted with cold GDP-fucose when FucT-VII was used as the enzyme source; lane 3, autoradiograph of [^{14}C]-labeled sY^2 reacted with cold GDP-fucose when crude Colo 205 cell homogenates were used as the enzyme source.

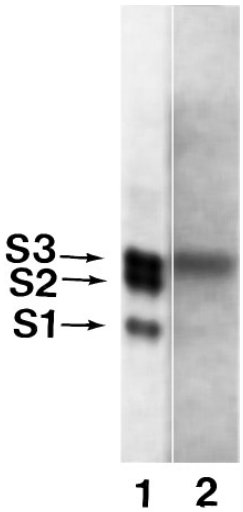

FIG. 2. HPTLC analysis of the monofucosylated derivative formed from $snLc_6$ by recombinant FucT-VII. Reaction products were prepared using the conditions described under Materials and Methods. Lane 1, autoradiograph of standards prepared by ^{14}C-fucose transfer by crude HL60 cell homogenate to nLc_6 using taurodeoxycholate as detergent (**12**). S1, $III^3V^3Fuc_2nLc_6$; S2, $III^3FucnLc_6$; S1, $V^3FucnLc_6$. Lane 2, autoradiograph of the monofucosylated derivative formed from $snLc_6$ by recombinant FucT-VII after desialylation of the isolated product by acidic acid. The solvent system was composed of *n*-propanol:H_2O:NH_4OH (7:3:1).

labeled sY^2 to a difucosyl derivative by transfer of unlabeled fucose from GDP-fucose, no difucosyl product was detectable using similar amounts of FucT-VII activity.

Two potential fucosylation sites exist when $snLc_6$ is used as a substrate. Fucosylation of $snLc_6$ may generate either of the monofucosylated derivatives $VI^3NeuAcV^3FucnLc_6$ or $VI^3NeuAcIII^3FucnLc_6$. Desialylation of the isolated monofucosylated product followed by TLC in n-propanol:H_2O:NH_4OH (7:3:1) (a solvent system that resolves the two potential monofucosylated derivatives) (**12**) resulted in a single band that co-migrated with the standard $V^3FucnLc_6$ (Figure 2). This indicates that the sole monofucosylated product generated by fucose transfer to $VI^3NeuAcnLc_6$ catalyzed by FucT-VII is $VI^3NeuAcV^3FucnLc_6$, a monofucosylated derivative of extended sLe^x.

DISCUSSION

The promyelocytic cell line HL60 strongly binds to E-selectin (**15,16**), and the ligands recognized by E-selectin in HL60 cells have been identified as monosialylated, multi-fucosylated, polylactosamine structures (**9,10**) (see Table II). Complete structural characterization of the HL-60 cell monosialylganglioside fraction revealed the absence of any monofucosylated sLe^x species (**9,10**). Strong evidence indicates that FucT-VII plays an important role in catalyzing the synthesis of E-selectin ligands (**8**), and it is well known that it catalyzes the synthesis of sLe^x (**6,7**); however, the fucosyltransferase(s) responsible for the biosynthesis of these structures in HL60 cells is unclear. At present, five human $\alpha1\rightarrow3$fucosyltransferases are known to exist. Each exhibits a unique characteristic with regard to the degree of substrate specificity. The results presented in this study indicate that FucT-VII displays the most restricted pattern of fucosylation when compared to other members of the $\alpha1\rightarrow3$fucosyltransferase family. FucT-VII is capable of catalyzing the transfer of only one fucose residue to a sialylated, lactosamine-based glycosphingolipid having multiple fucosylation sites, and the position of fucose transfer is restricted to the GlcNAc residue nearest to the negatively charged sialic acid. Transfer of fucose to neutral acceptors does not occur. This is in striking contrast to the substrate specificity of the FucT-III enzyme. FucT-III can utilize both type 1 (Gal$\beta1\rightarrow3$GlcNAc$\beta1\rightarrow$R) and type 2 (Gal$\beta1\rightarrow4$GlcNAc$\beta1\rightarrow$R) acceptors, transferring fucose in $\alpha1\rightarrow4$ or $\alpha1\rightarrow3$ linkage respectively, regardless of terminal galactose substitutions by either $\alpha1\rightarrow2$ linked fucose or $\alpha2\rightarrow3/6$ linked sialic acid (**14**). In addition, FucT-III can catalyze the incorporation of multiple fucose residues into polylactosamine structures (**14**). FucT-V and FucT-VI can transfer fucose efficiently to both neutral and sialylated type 2 chain structures, synthesizing both Le^x and sLe^x respectively (**5,17**); however, FucT-VI is unable to initially fucosylate the internal GlcNAc residue of extended lactosamine structures and thus is incapable of generating the VIM-2 epitope (**17**). FucT-IV has a more restricted acceptor specificity transferring fucose more efficiently to neutral type 2 chain acceptors than to sialylated acceptors (**12**). Fucosylation catalyzed by the HL60 cell derived enzyme (primarily FucT-IV) occurs for the most part on the internal GlcNAc residue when sialylated type 2 chain substrates with two lactosamine repeats were used, synthesizing the VIM-2 structure (**12**).

Northern blot analysis of mRNA isolated from HL60 cells revealed the presence of only FucT-IV and FucT-VII transcripts (**18**), thereby ruling out FucT-III, FucT-V, and FucT-VI as potential enzymes involved in fucosylating HL60 cell gangliosides. Results described herein indicate that FucT-VII is restricted to catalyzing the synthesis of only monofucosyl derivatives of sLe^x. Previous structural studies indicate the presence of additional fucosyl substitutions on HL60 cell gangliosides, some of which occur only on internal GlcNAc residues (**9,10**). Thus, biosynthesis of the E-selectin binding fractions from HL60 cells depends heavily on the action of FucT-IV along with the contribution from FucT-VII. Given the restricted ability of purified (**4**) or recombinant (**5**) FucT-IV to catalyze fucose transfer to sialylated acceptors, FucT-VII is most probably the key enzyme involved in fucose transfer to GlcNAc residues nearest to the sialic acid of neolacto-series acceptors in cells of myeloid origin.

TABLE II

Structures of Monosialylgangliosides from HL60 Cells

NeuAcα2-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R
NeuAcα2-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4[**Fucα1-3**]GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-R

Note. R, ceramide.

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