Glycobiology vol. 20 no. 9 pp. 1077–1088, 2010
doi:10.1093/glycob/cwq068
Advance Access publication on May 6, 2010

Helicobacter hepaticus Hh0072 gene encodes a novel α1-3-fucosyltransferase belonging to CAZy GT11 family

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Received on April 3, 2010; revised on April 29, 2010; accepted on April 29, 2010

Lewis x (Le^x) and sialyl Lewis x (SLe^x)-containing glycans play important roles in numerous physiological and pathological processes. The key enzyme for the final step formation of these Lewis antigens is α1-3-fucosyltransferase. Here we report molecular cloning and functional expression of a novel *Helicobacter hepaticus* α1-3-fucosyltransferase (HhFT1) which shows activity towards both non-sialylated and sialylated Type II oligosaccharide acceptor substrates. It is a promising catalyst for enzymatic and chemoenzymatic synthesis of Le^x, sialyl Le^x and their derivatives. Unlike all other α1-3/4-fucosyltransferases characterized so far which belong to Carbohydrate Active Enzyme (CAZy, http://www.cazy.org/) glycosyltransferase family GT10, the HhFT1 shares protein sequence homology with α1-2-fucosyltransferases and belongs to CAZy glycosyltransferase family GT11. The HhFT1 is thus the first α1-3-fucosyltransferase identified in the GT11 family.

Keywords: cloning/fucosyltransferase/*Helicobacter hepaticus*/Lewis x/sialyl Lewis x

Introduction

Fucosyltransferases (EC 2.4.1.-) are enzymes that catalyze the transfer of monosaccharide L-fucose from its activated sugar nucleotide guanosine-5′-diphosphate β-L-fucose (GDP-Fuc) to acceptors for the formation of fucosides (fucose-containing oligosaccharides and glycoconjugates). Mechanistically, fucosyltransferases are inverting glycosyltransferases (GTs) as α-fucosylated products are formed from β-fucosylated sugar nucleotide donor GDP-Fuc. Based on the types of acceptors and the regio-specificity of the fucosides formed by fucosyltransferase-catalyzed reaction, fucosyltransferases are categorized into α1-2, α1-3 and/or α1-4, α1-6 and *O*-fucosyltransferases (Ma et al. 2006). Except for *O*-fucosyltransferases (*O*-FucT) which catalyze the transfer of a fucose residue from GDP-Fuc directly to the serine or threonine residue on proteins (Ma et al. 2006), all others catalyze the transfer of a fucose residue to galactose (Gal), *N*-acetylglucosamine (GlcNAc) or another fucose (Fuc) residue (Marques et al. 1998) on oligosaccharides, polysaccharides or glycoconjugates. Furthermore, based on their protein sequence similarity, except for an unusual α1-2FucT in *Dictyostelium discoideum* (van Der Wel et al. 2001), all other α1-2FucTs are categorized into **C**arbohydrate **A**ctive En**zy**me (CAZy, http://www.cazy.org/) (Cantarel et al. 2009) glycosyltransferase family GT11, while all α1-3 and/or α1-4-FucTs characterized so far belong to GT10 (Ma et al. 2006).

L-Fucose is usually a terminal monosaccharide in oligosaccharides and the carbohydrate moieties of glycoconjugates. Protein *O*-fucosylation on serine or threonine has also been found in many plasma glycoproteins and is believed to be important for regulating protein functions including Notch signaling (Okajima et al. 2008). Fucose-containing structures in eukaryotes are believed to be involved in tissue development, angiogenesis, fertilization, cell adhesion, inflammation and tumor metastasis (Ma et al. 2006; Miyoshi et al. 2008). On the other hand, fucose-containing lipopolysaccharides (LPS) are expressed by some pathogenic bacteria including *Helicobacter pylori* and *Escherichia coli* (Guo et al. 2005). They have been suggested to be involved in molecular mimicry, adhesion, colonization and modulating the host immune response (Ma et al. 2006).

Lewis x (Le^x) trisaccharide Galβ1-4(Fucα1-3)GlcNAc and sialyl Lewis x (SLe^x) tetrasaccharide Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc are among the most important fucose-containing oligosaccharides that play essential roles in numerous physiological and pathological processes. For example, Le^x antigens expressed by human pathogenic bacterium *H. pylori* mimic the host cell antigens and mask the pathogenic bacterium from the host immune detection (Chan et al. 1995; Moran et al. 1996; Monteiro et al. 1998). Le^x antigens on parasite *Schistosoma mansoni* downregulate the host's protective immune response against the parasite (Wang et al. 2009). SLe^x is the essential recognition component of E-, P-, and L-selectin ligands (Kannagi 2002; Lowe 2003; Dube and Bertozzi 2005). The interaction of SLe^x and selectins is believed to mediate lymphocyte homing, initiate leukocyte–endothelial cell adhesion in acute and chronic inflammation (Kannagi 2002; Lowe 2003)

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and be involved in cancer migration (Ma et al. 2006). SLe^x is considered a cancer marker for diagnosis and prognosis of cancer metastasis (Kannagi 2004; Magnani 2004). It has been a leading structure for developing anti-inflammatory reagent and cancer vaccine (Simanek et al. 1998; Danishefsky and Allen 2000; Ouerfelli et al. 2005; Seeberger and Werz 2007).

Due to the importance of fucosides in biological systems and their potential application in treating inflammation, bacterial and viral infection and cancer, they have been attractive synthetic targets. Chemical synthesis of fucosidic bond is challenged by its acid lability; thus, fucosyltransferase-catalyzed enzymatic and chemoenzymatic approaches are considered preferred alternatives. Although many fucosyltransferases have been identified from humans, *Caenorhabditis elegans*, plants and bacteria (Ma et al. 2006), only *H. pylori* fucosyltransferases and human recombinant α1-3-fucosyltransferase have been used for the preparative-scale synthesis of Le^x (Lubineau et al. 1997, 1998; Wang et al. 2009) and SLe^x (Ichikawa et al. 1992; Bowman et al. 2001; Belot et al. 2002; Pratt and Bertozzi 2004). As the expression levels of reported *H. pylori* and human α1-3-fucosyltransferases are not high, there is a need for obtaining active and soluble α1-3-fucosyltransferases with high expression level, preferably in *E. coli* expression hosts, to allow large-scale enzymatic synthesis of fucosides.

H. pylori and *Helicobacter hepaticus* are two well-studied Gram-negative *Helicobacter* species. Unlike *H. pylori*, which is a gastric *Helicobacter* species, *H. hepaticus* is an enterohepatic *Helicobacter* species which colonizes the intestinal tracts and livers of mice to cause chronic hepatic inflammation, liver cancer and inflammatory bowel disease (Fox et al. 1994; Cahill et al. 1997). The association of *H. hepaticus* infection with liver tumors in mice provides a valuable animal model for studying mechanisms of liver cancers caused by bacterial infection (Rogers and Fox 2004). Like its close relative *H. pylori*, which can also cause chronic inflammation and carcinoma in the host (Suerbaum and Michetti 2002), *H. hepaticus* exhibits phase variation in genes encoding putative fucosyltransferases and other glycosyltransferases, which may contribute to LPS modification and antigenic mimicry (Wang et al. 2000) for efficient immune evasion of hosts (Suerbaum et al. 2003). Nevertheless, unlike fucosyltransferases from *H. pylori* (Chan et al. 1995; Monteiro et al. 1998; Ma et al. 2003, 2005; Rabbani et al 2005; Sanabria-Valentin et al. 2007; Sun et al. 2007, Wang et al. 2009), which have been well studied, no fucosyltransferase from *H. hepaticus* has been functionally characterized so far. Analysis of *H. hepaticus* ATCC51449 genome (Suerbaum et al. 2003) identified two genes (*HH0072* and *HH1776*) encoding putative fucosyltransferases in addition to two putative truncated α1-2-fucosyltransferase mutants (encoded by *HH0069* and *HH0070*). We report herein the cloning, functional expression and characterization of the first fucosyltransferase (HhFT1) from *H. hepaticus* encoded by gene *HH0072* as a novel α1-3-fucosyltransferase which can use both sialylated (Neu5Acα2-3Galβ1-4GlcNAc) and non-sialylated (Galβ1-4GlcNAc) type II glycans as acceptors for the synthesis of sialyl Lewis x [Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc] and Lewis x [Galβ1-4(Fucα1-3)GlcNAc] antigens. It is the first α1-3-fucosyltransferase identified in CAZy glycosyltransferase family GT11, which contains, so far, only α1-2-fucosyltransferases. The cloning and characterization of the second putative *H. hepaticus* fucosyltransferase (HhFT2) encoded by gene *HH1776* are ongoing processes.

Results

Sequence comparison of HhFT1, HhFT2 and other bacterial fucosyltransferases

Proteins encoded by *HH0072* (GenBank accession number AAP76669) and *HH1776* (GenBank accession number AAP78373) in carcinogenic bacterium *H. hepaticus* ATCC 51449 genome are annotated as conserved hypothetical proteins predicted to be a fucosyltransferase and an α1-3-fucosyltransferase, respectively. Here, we name them HhFT1 and HhFT2, respectively. Based on protein sequence homology, HhFT1 encoded by *HH0072* is classified into CAZy glycosyltransferase family GT11 which contains all α1-2-fucosyltransferases characterized so far except for an unusual α1-2FucT in *D. discoideum* (van Der Wel et al. 2001). HhFT2 encoded by *HH1776* is classified into CAZy glycosyltransferase family GT10 which contains all α1-3/4-fucosyltransferases characterized so far. Nevertheless, they are quite divergent from other characterized bacterial fucosyltransferases (Figure 1). The protein which has the closest sequence homology to HhFT1 is an *E. coli* O128:B12 α1-2-fucosyltransferase WbsJ (GenBank accession number AAO37698) which is a part of *O*-antigen biosynthesis cluster (Shao et al. 2003). They share 27% identity and 50% similarity. Sequence alignment (Figure 2) indicates that HhFT1 has three conserved motifs shared by GT11 family fucosyltransferases including motif II which is likely a part of the GDP-Fuc binding domain (Shao et al. 2003). The protein which has the closest sequence homology to HhFT2 is a *H. pylori* α1-4-fucosyltransferse (GenBank accession number AAR88243) (Rabbani et al. 2005), and they share 35% identity and 51% similarity. Sequence-based protein solubility prediction (Smialowski et al. 2007) indicates that HhFT1 has a better solubility score than all other possible fucosyltransferases except for a putative protein from *Geobacter lovleyi* SZ (GenBank accession number ACD96461). Unlike α1-3FucTs from *H. pylori* (Ge et al. 1997; Lin et al. 2006), HhFT1 does not have C-terminal tandem repeats, and it is a shorter protein (320 amino acid residues) compared to the full-length Hp1-3FT (478 amino acid residues) (Lin et al. 2006).

Cloning, expression and purification of recombinant protein

Full-length *H. hepaticus HH0072* synthetic gene with codons optimized for *E. coli* expression was initially cloned into pET15b vector as an N-His_6-tagged recombinant protein (His_6-HhFT1), but a low amount of soluble protein was obtained in cell lysate (less than 1 mg/L cell culture, data not shown). In order to increase the amount of soluble recombinant protein, pMAL-c4X vector (Kapust and Waugh 1999) was chosen to express HhFT1 as a maltose binding protein (MBP)-fusion protein with the MBP tag at the N-terminus. A His_6-tag was also introduced at the C-terminus to allow easy purification of the recombinant protein by Ni^{2+}-affinity columns. The gene and protein sequences of the resulting fusion protein MBP–HhFT1–His_6 are shown in Figure 3. The codon-

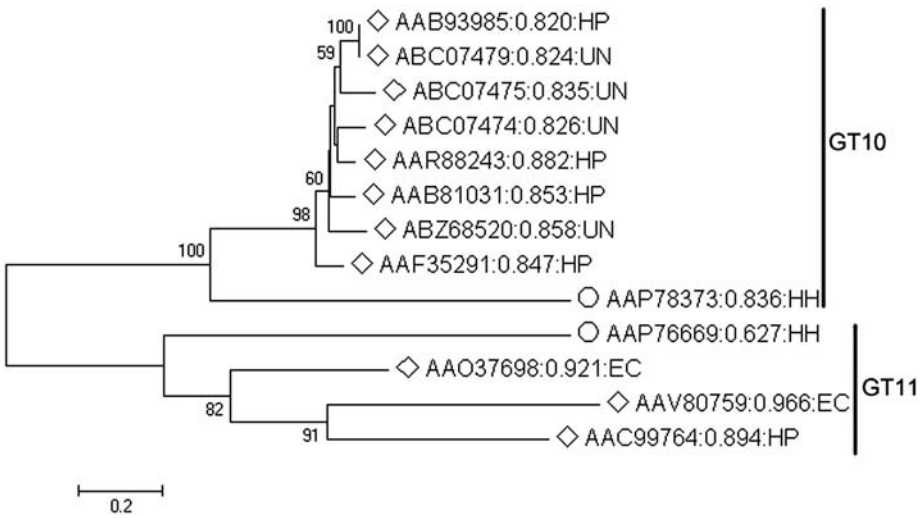

Fig. 1. Phylogenetic analysis of identified (labeled with unfilled diamonds) and putative (labeled with unfilled circles) FucTs from bacteria. Neighbor-Joining (NJ) method was used to create this unrooted tree. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are indicated above the branches. The protein identification numbers are followed by predicted class probability of insolubility (Smialowski et al. 2007) and species from which the genes were cloned. HP, *H. pylori*; EC, *E. coli*; HH, *Helicobacter hepaticus*; UN, unknown.

optimized *HhFT1* gene contains 27% adenine, 18% cytosine, 22% guanine and 33% thymine as compared to the original sequence containing 29% adenine, 19% cytosine, 21% guanine and 31% thymine (GenBank accession no. AE017125). Expression of the recombinant protein MBP–HhFT1–His_6 in *E. coli* BL21 (DE3) followed by Ni^{2+}-column purification resulted in a yield of 45 mg L^{-1} cell culture. This expression level is the highest among all reported recombinant fucosyltransferases. As shown in Figure 4, the purified protein exhibited a molecular mass of about 75 kDa in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) which is close to the calculated molecular weight of 80 kDa.

Acceptor substrates of MBP–HhFT1–His_6

Using GDP-fucose as a donor, both type I (Galβ1-3GlcNAcβ-$ProN_3$) and type II (Galβ1-4GlcNAcβ$ProN_3$) disaccharides were tested as potential acceptor substrates for the recombinant MBP–HhFT1–His_6. Thin-layer chromatography (TLC) analyses indicated that the recombinant enzyme was reactive towards type II Galβ1-4GlcNAcβ$ProN_3$ substrate, and no product was observed when type I disaccharide Galβ1-3GlcNAcβ$ProN_3$ was used as the potential acceptor substrate. Further TLC tests indicated that α2-3-sialylated type II oligosaccharide with *N*-acetylneuraminic acid (Neu5Ac) as the sialic acid form (Neu5Acα2-3Galβ1-4GlcNAcβ$ProN_3$) was also an acceptor for the enzyme, suggesting that this novel recombinant enzyme is most likely an α1-3-fucosyltransferase in spite of its protein sequence homology to α1-2-fucosyltransferases in CAZy glycosyltransferase family GT11.

Confirming the α1-3-fucosyltransferase activity of MBP–HhFT1–His_6 by one-pot two-enzyme synthesis of Galβ1-4(Fuc1-3)GlcNAcβ$ProN_3$

In order to confirm the α1-3-fucosyltransferase activity of MBP–HhFT1–His_6, a preparative-scale synthesis of Lewis x trisaccharide Galβ1-4(Fucα1-3)GlcNAcβ$ProN_3$ from type II disaccharide Galβ1-4GlcNAcβ$ProN_3$ was carried out using a one-pot two-enzyme reaction (Figure 5) containing the recombi-

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HhFT1 : MKDDLVILHPDGGIASQIAFVALGLAFEQKGAKVKYDLSWFAEGAKGFWNPSNGYDKVYDITWDISKA :  68
WbsJ  : -----MEVKIIGGLGNQMFQYATAFAIAKR---THQNLTVDISDAVKYKTHPLRLVELSCSSEFVKKA :  60

HhFT1 : FPALHIEIANEEEIERYKSKYLIDN---DRVIDYAPPLYCYGYKGRIFHYLYAPFFAQSFAPKEAQDS : 133
WbsJ  : WPFEKYLFS--EKIPHFMKKGMFRKHYVEKSLEYDPDIDTKSINKKIVGYFQTEKYFKEFRHELIKEF : 126

HhFT1 : H-----TPFAALLQEIESSPSPCGVHIRRGDLSQPHIVYGNP--TSNEYFAKSIELMC---LLHPQSS : 191
WbsJ  : QPKTKFNSYQNELLNLIKENDTCSLHIRRGDYVSSKIANETHGTCSEKYFERAIDYLMNKGVINKKTL : 194

HhFT1 : FYLFSDDLAFVKEQIVPLLKGKTYRICDVN-NPSQGYLDLYLLSRCRNIIGSQGSMGEFAKVLSPHNP : 258
WbsJ  : LFIFSDDIKWCRENIF-----FNNQICFVQGDAYHVELDMLLMSKCKNNIISNSSFSWWAAWLNENKN : 257

HhFT1 : LLITPRYRNIFKEVEN-VMCVNWGESVQHPPLVCSAPPPLVSQLKRNAPLNSRLYKEKDNASA : 320
WbsJ  : KTVIAPSKWFKKDIKHDIIPESWVKL---------------------------------- : 283
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Fig. 2. Alignment of HhFT1 and *E. coli* O128:B12 α1-2-fucosyltransferase WbsJ (GenBank accession number AAO37698).

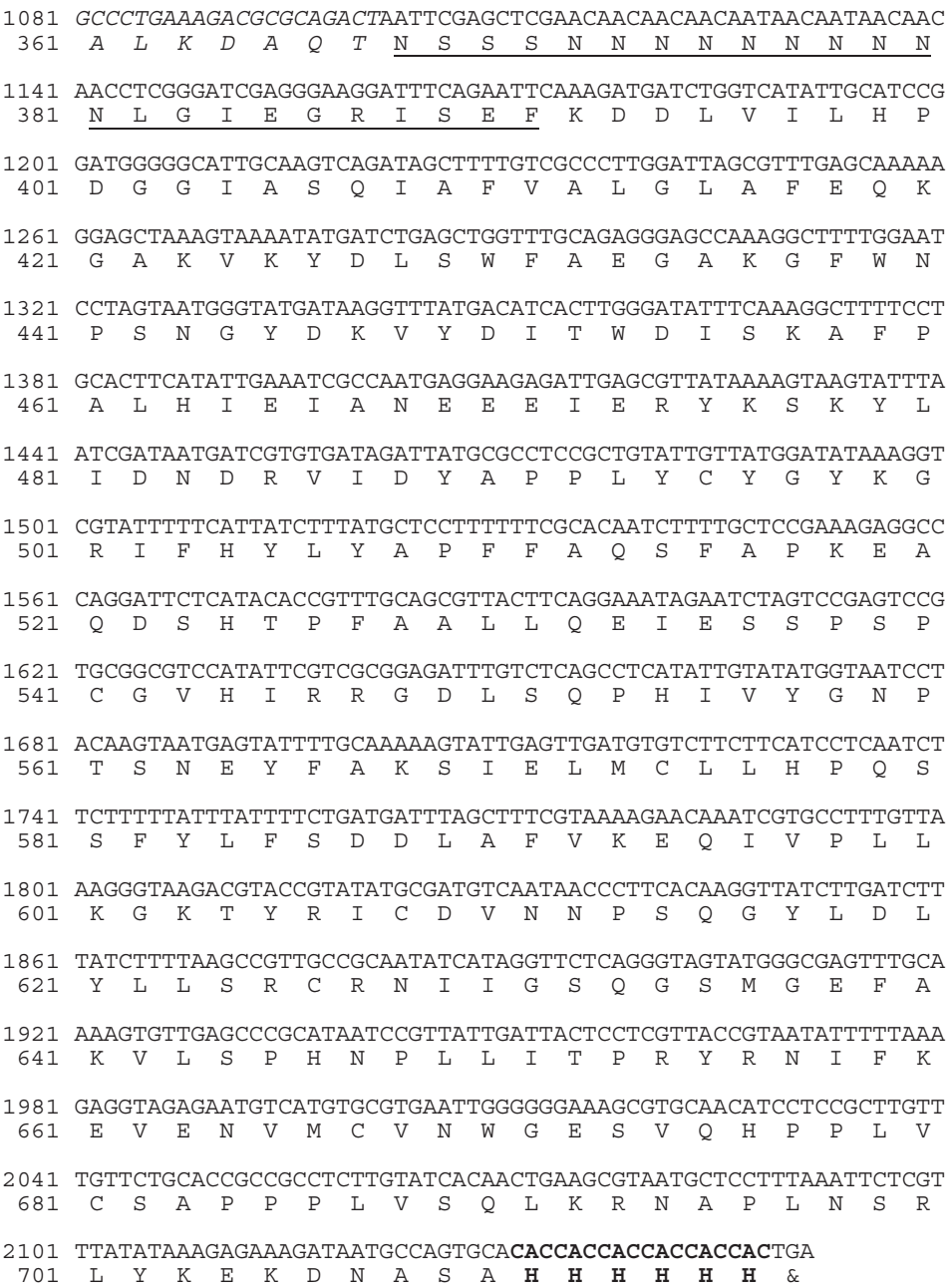

Fig. 3. Gene and protein sequences of the codon-optimized MBP–HhFT1–His_6. Only the C-terminal sequence of MBP is shown (in italics). The multiple cloning sites of pMAL-c4X vector are underlined. The six histidine residues introduced at the C-terminus during cloning are in bold.

nant fucosyltransferase MBP–HhFT1–His_6 and a recombinant bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (FKP) from *Bacteroides fragilis* strain NCTC9343 (Yi et al. 2009). The presence of the bifunctional FKP allows the synthesis of GDP-fucose, the sugar nucleotide donor for MBP–HhFT1–His_6, from simple starting materials such as L-fucose, adenosine 5′-triphosphate (ATP) and guanosine-5′-triphosphate (GTP) (Yi et al. 2009). Lewis x (Le^x) trisaccharide Galβ1-4 (Fucα1-3)GlcNAcβProN_3 was obtained in a yield of 63% with the supplement of additional ATP and GTP periodically (see Materials and methods for details). Nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) studies confirmed the structure of the trisaccharide product. As shown in Table I, the ^{13}C NMR chemical shifts of the purified product agree well with previously reported data for Lewis x trisaccharide (Wang et al. 2009). More specifically, comparing the chemical shifts of the disaccharide starting material and the trisaccharide product indicates that significant changes occur at C-3 (a downfield shift of 2.35 ppm from 72.70 ppm in the disaccharide starting material to 75.05 ppm in the trisaccharide product) and C-4 (an upfield shift of 3.22 ppm from 78.69 ppm in the disaccharide starting material to 75.47 ppm in the trisaccharide product) of the GlcNAc residue, confirming that fucosylation takes place at C-3 of the GlcNAc residue. High-

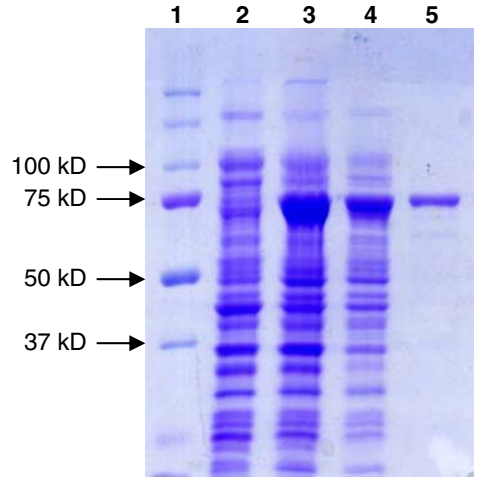

Fig. 4. SDS-PAGE analysis of MBP–HhFT1–His_6 expression and purification. Lanes: 1, protein standards; 2, whole cell extraction before induction; 3, whole cell extraction after induction; 4, cell lysate after induction; 5, Ni^{2+}-column purified protein.

resolution mass spectrometry spectrum (ESI) obtained shows the desired *m/z* for molecular ion $[M]^{+\cdot}$ of 612.2425 (calculated 612.2490 for $C_{23}H_{40}N_4O_{15}$).

pH Profile of MBP–HhFT1–His_6

High-performance liquid chromatography (HPLC)-based pH profile study for the fucosyltransferase was carried out using fluorophore 2-anthranilic acid (2AA)-labeled non-sialylated and sialylated type II oligosaccharides (Galβ1-4GlcNAcβ-Pro2AA and Neu5Acα2-3Galβ1-4GlcNAcβPro2AA respectively) as acceptors. A similar pH profile pattern was seen for the MBP–HhFT1–His_6 with these two acceptors, although the overall activity was higher when the sialylated acceptor Neu5Acα2-3Galβ1-4GlcNAcβPro2AA was used (Figure 6). The fucosyltransferase activity was optimum in a relatively broad pH range of 6.0–8.0. Medium activity was observed at pH 5.5 and in a pH range of 8.5–9.0. No or minimum activity was observed when the pH fell below 5.0 or higher than 9.5. This pH profile is similar to that reported recently for an *E. coli* O128:B12 α1-2-fucosyltransferase WbsJ, which is also active in a wide range of pH values (5.5–8.5) (Li et al. 2008).

Table I. ^{13}C NMR chemical shifts assignment of LacNAcβProN_3 (Galβ1-4GlcNAcβProN_3) and Le^xβProN_3 [Galβ1-4(Fuc1-3)GlcNAcβProN_3]

Residue βDGlcNAc	Carbon atom C	Chemical shift (ppm)	
		LacNAcβProN_3	Le^xβProN_3
	1	101.27	101.09
	2	55.26	55.94
	3	**72.70**	**75.05**
	4	**78.69**	**75.47**
	5	74.91	73.49
	6	60.26	59.88
	C=O	174.61	174.40
	CH_3	22.37	22.36
βDGal(1-4)	1	103.07	101.96
	2	71.14	71.16
	3	72.54	72.58
	4	68.72	68.47
	5	75.52	75.10
	6	61.19	61.63
αLFuc(1-3)	1		98.78
	2		67.82
	3		69.33
	4		72.03
	5		66.85
	CH_3		15.43
ProN_3	$OCH_2CH_2CH_2N_3$	67.29	67.32
	$OCH_2CH_2CH_2N_3$	28.29	28.24
	$OCH_2CH_2CH_2N_3$	47.96	47.88

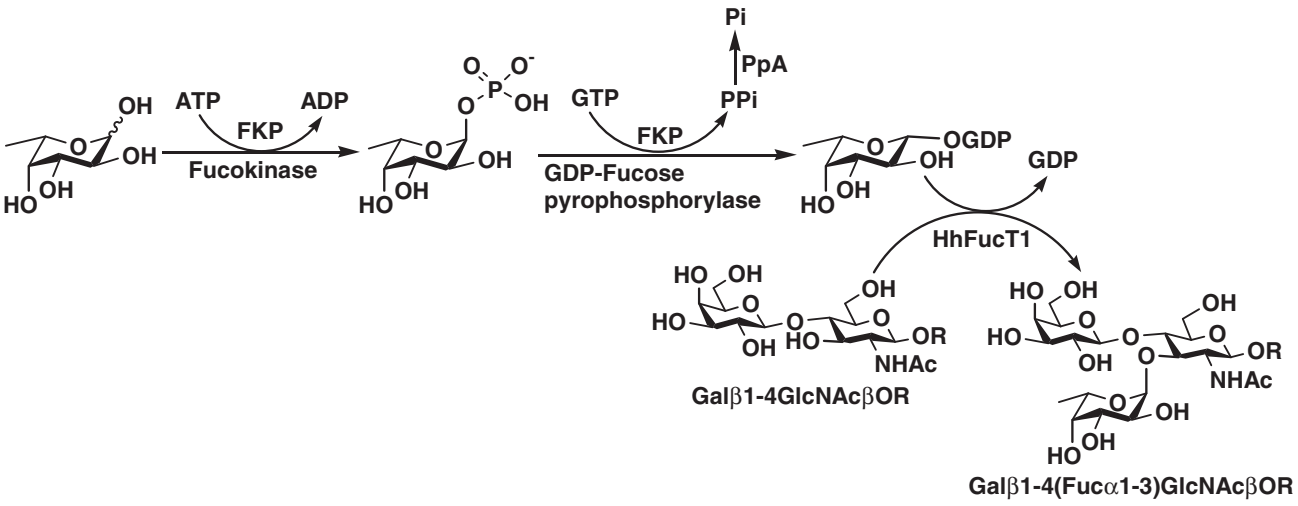

Fig. 5. Schematic illustration for the one-pot three-enzyme preparative-scale synthesis of Lewis x trisaccharide Galβ1-4(Fucα1-3)GlcNAcβProN_3 from fucose, ATP, GTP and type II disaccharide Galβ1-4GlcNAcβProN_3 (LacNAcβProN_3). Enzymes used: FKP, a recombinant bifunctional L-**fuco**kinase/GDP-fucose **p**yrophosphorylase from *B. fragilis* strain NCTC9343; HhFT1, ***H. hepaticus*** α1-3-**fuco**syl**t**ransferase **1**; PpA, a recombinant inorganic **pyro**phospha**t**ase from *P. multocida*.

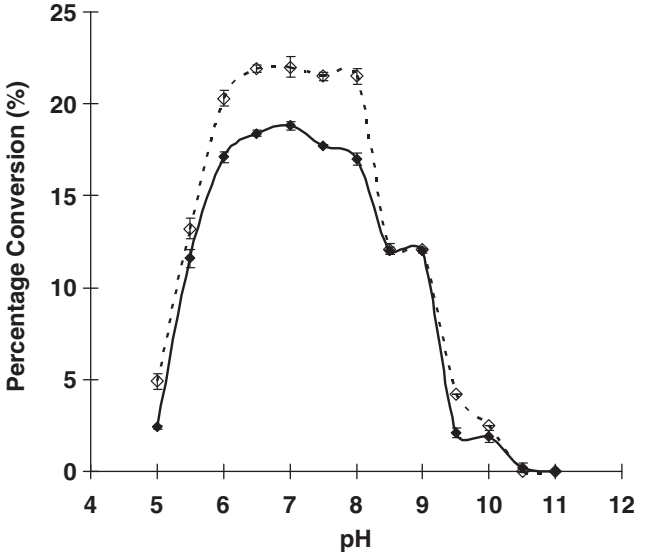

Fig. 6. The pH profile of MBP–HhFT1–His_6 when LacNAcβPro2AA (filled diamonds) or Neu5Acα2-3LacNAcβPro2AA (dashed line with unfilled diamonds) was used as an acceptor substrate. Buffers used: MES (pH 5.0–6.5), HEPES (pH 7.0–8.0), Tris-HCl (pH 8.5–9.0), *N*-cyclohexyl-2-hydroxyl-3-aminopropanesulfonic acid (CAPSO) (pH 10), and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) (pH 10.0–11.0).

Effects of metal ions, EDTA and dithiothreitol on the fucosyltransferase activity of MBP–HhFT1–His_6

The effects of dithiothreitol (DTT), metal ions Mg^{2+} and Mn^{2+}, and a chelating agent EDTA on the fucosyltransferase activity of MBP–HhFT1–His_6 were examined at pH 7.0. As shown in Figure 7, a divalent metal ion is required for the fucosyltransferase activity of the enzyme as the enzyme activity decreased dramatically in the absence of metal ions. This is consistent with the requirement of the divalent metal ion by most glycosyltransferases using nucleotide diphosphate sugars as donor substrates. The enzyme activity increases with the increase of the metal ion concentration up to 50 mM. The divalent metal ions may facilitate the glycosidic bond cleavage in the donor by neutralizing the negative charges on the GDP product. Indeed, protein sequence analysis of HhFT1 indicates a metal-binding $D^{92}ND^{94}$ motif. It is also noticed that the enzyme activity did not disappear completely without the addition of metal ions or in the presence of a metal chelator EDTA. The reason is unclear, but it is possible that positively charged amino acid residues in the protein may contribute to stabilize the GDP formed (Sun et al. 2007).

There are seven cysteine residues in the HhFT1 protein sequence, so the effect of DTT on the fucosyltransferase activity of the enzyme was studied. The addition of DTT did not change the enzyme activity significantly, indicating that disulfide formation is not required for the fucosyltransferase activity of MBP–HhFT1–His_6.

Kinetics

Kinetic studies carried out for the fucosyltransferase activity of MBP–HhFT1–His_6 using sialylated and non-sialylated type II acceptors indicate that both glycans are similarly good acceptors for the enzyme although the efficiency for sialylated glycan Neu5Acα2-3LacNAcβPro2AA (k_{cat}/K_m = 0.63) is a little better than the non-sialylated glycan LacNAcβPro2AA (k_{cat}/K_m = 0.43) due to a smaller K_m and a better k_{cat} for the sialylated acceptor (Table II). This is different from a C-terminal truncated α1-3-fucosyltransferase (HpFTΔ45) cloned from *H. pylori* (NCTC 11639) (Lin et al. 2006) which prefers *N*-acetyllactosamine (LacNAc) over Neu5Acα2-3LacNAc as an acceptor. Nevertheless, the K_m value of LacNAcβPro2AA (45 mM) for MBP–HhFT1–His_6 is about 50-fold higher than that (0.71–0.89 mM) of LacNAc for truncated α1-3-fucosyltransferases cloned from *H. pylori* (NCTC 11639) (Lin et al. 2006), indicating a much weaker binding of the type II glycan to the HhFT1 than the *H. pylori* FucT.

GDP-fucose hydrolysis activity of the MBP–HhFT1–His_6

In the absence of a glycan acceptor, water can be considered as an acceptor for the fucosyltransferase activity of the enzyme. This leads to the GDP-fucose hydrolysis. Significant GDP-fucose hydrolysis activity was observed for MBP–HhFT1–His_6 with an optimal activity at pH 8.0 (Figure 8). At pH 7.0, the hydrolysis of GDP-fucose (k_{cat}/K_m = 3.7) catalyzed by the enzyme is about 3-fold more efficient than the fucosyltransferase activity of the enzyme when either non-sialylated (k_{cat}/K_m = 1.0) or sialylated type II glycans (k_{cat}/K_m = 1.1) was used as an acceptor (Table II).

Fucosidase activity studies

No fucosidase activity was observed for the MBP–HhFT1–His_6 when Le^xβProN_3 was used as a substrate for TLC assays or when 4-methylumbelliferyl β-Lewis x (Le^xβMU) and Neu5Acα2-3Le^xβMU were used in HPLC-based assays.

Product inhibition studies using GDP or Le^xβProN_3 as an inhibitor

No product inhibition effect was observed for GDP or Le^xβ-ProN_3 by HPLC-based fluorescent assays.

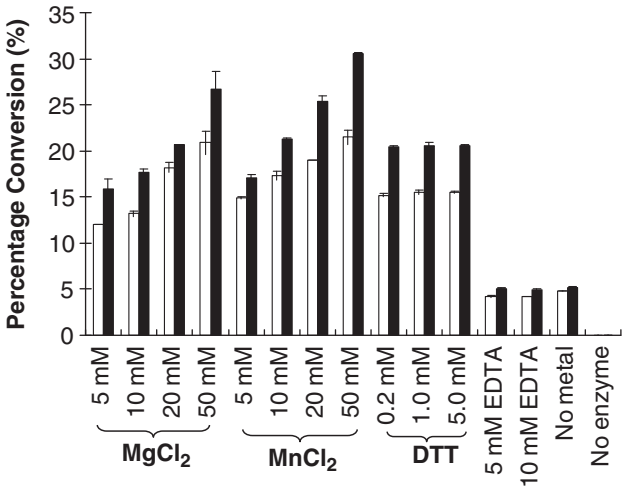

Fig. 7. Effects of metal ions (Mg^{2+} and Mn^{2+}), EDTA, and DTT (in the presence of 20 mM Mg^{2+}) on the fucosyltransferase activity of MBP–HhFT1–His_6 when LacNAcβPro2AA (white columns) or Neu5Acα2-3LacNAcβPro2AA (black columns) was used as an acceptor substrate.

Table II. Kinetic parameters of recombinant MBP–HhFT1–His_6

	Substrates	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Fucosyltransferase	LacNAcβPro2AA	45 ± 2	19 ± 1	0.43
	GDP-fucose	2.4 ± 0.4	2.3 ± 0.1	1.0
	Neu5Acα2-3LacNAcβPro2AA	40 ± 1	25 ± 1	0.63
	GDP-fucose	3.3 ± 0.6	3.6 ± 0.2	1.1
GDP-Fucose hydrolysis	GDP-fucose	13 ± 2	48 ± 2	3.7

Discussion

In this study, we report the molecular cloning, functional expression and characterization of a novel *H. hepaticus* α1-3-fucosyltransferase. Based on its protein sequence homology, HhFT1 is classified into glycosyltransferase family 11 (GT11) in the Carbohydrate Active enZyme database (CAZy, http://www.cazy.org/) (Campbell et al. 1997; Coutinho et al. 2003). All members of GT11 family that have been functionally determined so far are α1-2 fucosyltransferases. These include WbsJ from *E. coli* O128:B12 (Li et al. 2008), a potential α1-2FT WbwK from *E. coli* O86:B7 (Guo et al. 2005), HpFT2 from *H. pylori* (Wang et al. 1999), CE2FT-1 and CE2FT-2 (Zheng et al. 2008) from *C. elegans*, FUT1 and FUT2 from human (Larsen et al. 1990; Kelly et al. 1995; Rouquier et al. 1995; Kudo et al. 1996; Koda et al. 1997), mouse (Lin et al. 2000; Domino et al. 2001), rat (Piau et al. 1994; Bureau et al. 2001), bovine (Barreaud et al. 2000; Saunier et al. 2001), and primates (Apoil et al. 2000), as well as Sec1 from bovine (Barreaud et al. 2000; Saunier et al. 2001), mouse (Lin et al. 2000; Domino et al. 2001) and primates (Borges et al. 2008). The enzyme has the highest sequence homology to HhFT1 is WbsJ, an *E. coli* O128:B12 α1-2-fucosyltransferase which is a part of *O*-antigen biosynthesis cluster (Shao et al. 2003). It shares 27% identity and 50% similarity to HhFT1.

In comparison, all α1-3 and/or α1-4-fucosyltransferases characterized so far are listed in GT10 family. HhFT1 thus represents the first α1-3-fucosyltransferase that belongs to the CAZy GT11 family. This also indicates that, while protein sequence homology can be used as a guide to predict the type of the enzyme, experimental confirmation is necessary to characterize the function of the protein.

All fucosyltransferases are inverting glycosyltransferases and are classified into eight glycosyltransferase (GT) families in CAZy website: GT10, GT11, GT23, GT3, GT56, GT65, GT68 and GT74 (Campbell et al. 1997; Coutinho et al. 2003; Cantarel et al. 2009). The only known fucosyltransferase crystal structures are for *H. pylori* α1-3FT in the GT10 family (Sun et al. 2007) and *Bradyrhizobium* sp. WM9 α1-6FT NodZ (EC 2.4.1.-) (Brzezinski et al. 2007) and human GlcNAc α1-6FT (FUT8) (EC 2.4.1.68) (Ihara et al. 2007) in the GT23 family. All of these fucosyltransferases have a glycosyltransferase B (GT-B) fold containing two separated Rossmann domains (Breton et al. 2006). Protein x-ray crystal structures have not been reported for any member of the GT11 family. The good solubility and expression level (45 mg L^{-1} cell culture) of MBP–HhFT1–His_6 present an excellent opportunity for structural characterization of GT11 fucosyltransferases.

Similar to *H. pylori*, the hypothetic genes involved in LPS synthesis of *H. hepaticus* ATCC 51449 are scattered throughout the whole genome. Homologues of *HP0826* (β1-4-galactosyltransferase gene), *HP0360* (*galE*), *HP0326* (*neuA*) as well as GDP-fucose biosynthetic genes *rfbM* (*HP0043*), *rfbD* (*HP0044*) and *wbcJ* (*HP0045*), which are involved in the synthesis of Lewis antigen in *H. pylori* 26695 (Moran 2008), are also harbored by *H. hepaticus* ATCC 51449 (*HH0323*, *HH0380*, *HH0900*, *HH0675*, *HH0172* and *HH0173*, respectively). Analysis of *H. hepaticus* ATCC 51449 genome has identified two CMP-Neu5Ac pathway genes, *neuB* (*HH0908*) and *neuC* (*HH0082*) (Daines et al. 2000), although no sialyltransferase has been discovered by searching the whole *H. hepaticus* ATCC 51449 genome using known sialyltransferase sequences from uniproKB database (http://www.uniprot.org/). A putative sialidase (*HH1672*) is also found in the genome of *H. hepaticus* ATCC 51449.

GDP-fucose can be efficiently hydrolyzed by MBP–HhFT1–His_6, which competes with the fucosyltransferase activity of the enzyme, leading to low yields due to the consumption of the donor substrate by water (Figure 9). This can be overcome by adding additional GDP-fucose periodically during the reaction process. In situ generation of GDP-

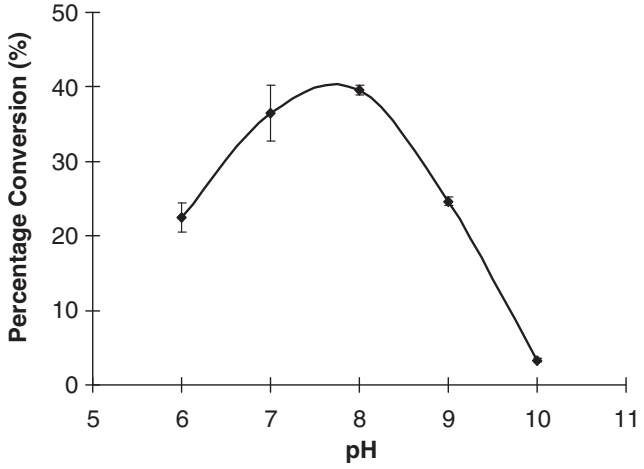

Fig. 8. The pH profile of GDP-fucose hydrolysis catalyzed by MBP–HhFT1–His_6. Buffers used: MES, pH 6.0; HEPES, pH 7.0, 8.0; Tris-HCl, pH 9.0; and CAPS, pH 10.0.

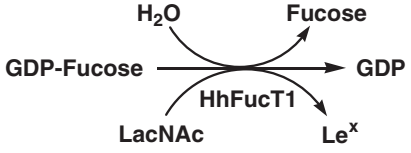

Fig. 9. Schematic illustration of water as a competing acceptor for the fucosyltransferase activity of MBP–HhFT1–His_6 causing hydrolysis of GDP-fucose.

fucose from GTP and ATP using a bifunctional FKP from *B. fragilis* strain NCTC9343 (Yi et al. 2009) also helps to improve the yield. As most glycosyltransferases can catalyze the hydrolysis of the corresponding sugar nucleotide donor substrates, periodical addition of sugar nucleotides and in situ generation of sugar nucleotides can be used as general strategies to improve the yields of glycosyltransferase-catalyzed reactions. These will be extremely useful approaches when weak acceptors are used and water competes significantly for the same donor.

Materials and methods

Materials

E. coli electrocompetent DH5α and chemically competent BL21 (DE3) cells were from Invitrogen (Carlsbad, CA, USA). Vector plasmid pET15b was purchased from Novagen (EMD Biosciences Inc. Madison, WI, USA). QIAprep spin miniprep kit and QIAquick gel extraction kit were from Qiagen (Valencia, CA, USA). Herculase-enhanced DNA polymerase was from Stratagene (La Jolla, CA, USA). T4 DNA ligase and 1 kb DNA ladder were from Promega (Madison, WI, USA). *Nde*I, *Bam*HI, *Eco*RI, *Hin*dIII restriction enzymes and vector plasmid pMAL-c4X were from New England Biolabs (Beverly, MA, USA). Ni^{2+}-NTA agarose (nickel-nitrilotriacetic acid agarose) was from 5 PRIME (Gaithersburg, MD, USA). Bicinchoninic acid (BCA) protein assay kit was from Pierce Biotechnology Inc. (Rockford, IL).

Chemical synthesis of GDP-fucose

GDP-fucose was synthesized by following a reported procedure (Timmons and Jakeman 2007). Detailed procedures are presented in the supporting information.

Synthesis of LacNAcβPro2AA and Neu5Acα2-3LacNAcβPro2AA as acceptors for HhFT1

Fluorescent LacNAcβPro2AA was synthesized from LacNAcβProNH$_2$ (obtained by reduction of azidoproyl LacNAc, LacNAcβProN$_3$) (Chokhawala et al. 2008) and 2-(methoxycarbonyl) succinanilic acid NHS ester (2AA-OSu). Its α2-3-sialylated form Neu5Acα2-3LacNAcβPro2AA was synthesized from LacNAcβPro2AA using a one-pot two-enzyme sialylation reaction (Yu et al. 2009) catalyzed by a *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS) (Yu et al. 2004) and a multifunctional *Pasteurella multocida* α2-3-sialyltransferase (PmST1) (Yu et al. 2005). Detailed procedures are presented in the supporting information.

Cloning

Full-length *H. hepaticus HH0072* synthetic gene with codons optimized for *E. coli* expression was customer synthesized by Biomatik (Wilmington, DE, USA) and provided in a pGH vector. It was cloned as an N-His$_6$-tagged or an N-MBP-tagged with a C-His$_6$-tagged fusion protein. The primers used for cloning the N-His$_6$-tagged protein in pET15b vector were: forward primer 5′-GATCCATATGAAAGATGATCTGGTCATATTGCAT-3′ (*Nde*I restriction site is underlined) and reverse primer 5′-AAGGGATCCTCATGCACTGGCATTATCTTTCTCTT-3′ (*Bam*HI restriction site is underlined). To clone the N-MBP-tagged with C-His$_6$-tagged protein in pMAL-c4X vector, the forward primer used was 5′-GATCGAATTCAAAGATGATCTGGTCATATTGCAT-3′ (*Eco*RI restriction site is underlined), and the reverse primer used was 5′-AAGAAGCTTTCA*GTGGTGGTGGTGGTGGTG*TGCACTGGCATTATCTTTCTCTT-3′ (*Hin*dIII restriction site is underlined, and the sequence that encodes the hexahistidine tag is italicized). Polymerase chain reactions (PCRs) for amplifying the target gene were performed in a 50-μL reaction mixture containing plasmid DNA (10 ng), forward and reverse primers (0.2 μM each), 1× Herculase buffer, dNTP mixture (0.2 mM), and 5 U (1 μL) of Herculase-enhanced DNA polymerase. The reaction mixture was subjected to 30 cycles of amplification at an annealing temperature of 55°C. The resulting PCR product was purified and double digested with *Nde*I and *Bam*HI or *Eco*RI and *Hind*III restriction enzymes. The purified and digested PCR product was ligated with the predigested pET15b or pMAL-c4X vector and transformed into electrocompetent *E. coli* DH5α cells. Selected clones were grown for minipreps and characterized by restriction mapping. DNA sequencing was performed by the Davis Sequencing Facility in the University of California-Davis.

Overexpression

Positive plasmids were selected and transformed into *E. coli* BL21 (DE3) chemical competent cells. The plasmid-bearing *E. coli* strains were cultured in LB-rich medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl) supplemented with ampicillin (100 μg mL^{-1}). Overexpression of the target protein was achieved by inducing the *E. coli* culture with 0.1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) when OD_{600} reached 0.8 followed by incubating at 20°C for 20 h with vigorous shaking at 250 rpm in a C25KC incubator shaker (New Brunswick Scientific, Edison, NJ).

Purification

His$_6$-tagged target proteins were purified from cell lysate. To obtain the cell lysate, cell pellet harvested by centrifugation at 4000 rpm for 2 h was resuspended in lysis buffer (pH 8.0, 100 mM tris(hydroxymethyl)aminomethane (Tris)-HCl containing 0.1% Triton X-100) (20 mL L^{-1} cell culture). Lysozyme (50 μg mL^{-1}) and DNaseI (3 μg mL^{-1}) were then added, and the mixture was incubated at 37°C for 60 min with vigorous shaking. Cell lysate was obtained by centrifugation at 12,000 rpm for 30 min as the supernatant. Purification of His-tagged proteins from the lysate was achieved using a Ni^{2+}-resin column. The column was pre-equilibrated with 10 column volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5) before the lysate was loaded. After washing with 8 column volumes of binding buffer and 10 column volumes of washing buffer (40 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5), the protein was eluted with an elute buffer (200 mM imidazole, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5). The fractions containing the purified enzymes were collected and stored at 4°C.

Quantification of purified protein

The concentration of purified enzyme was obtained in a 96-well plate using a BCA Protein Assay Kit (Pierce Biotechnology,

Rockford, IL) with bovine serum albumin as a protein standard. The absorbance of samples was measured at 562 nm by a BioTek Synergy™ HT Multi-Mode Microplate Reader.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed in a 12% Tris-glycine gel using a Bio-Rad Mini-protein III cell gel electrophoresis unit (Bio-Rad) at DC = 150 V. Bio-Rad Precision Plus Protein Standards (10–250 kDa) were used as molecular weight standards. Gels were stained with Coomassie Blue.

TLC analysis of the substrate specificity

LacNAcβProN$_3$ (Galβ1-4GlcNAcβProN$_3$, type II, 10 mM) or Galβ1-3GlcNAcβProN$_3$ (type I, 10 mM) was used as a potential acceptor in the presence of GDP-fucose (10 mM) in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.0, 100 mM) to analyze the acceptor substrate specificity of the fucosyltransferase activity of the recombinant enzyme (9.0 μg) at 37°C. The crude reaction mixture was analyzed by TLC.

One-pot two-enzyme synthesis of Galβ1-4(Fucα1-3) GlcNAcβProN$_3$ using MBP–HhFT1–His$_6$

HhFT1 acceptor Galβ1-4GlcNAcβProN$_3$ (30 mg), L-fucose (21 mg, 2 equivalents), ATP (71 mg, 2 equivalents), GTP (67 mg, 2 equivalents) and $MgCl_2$ (45 mg, 22 mM) were dissolved in H_2O. A stock solution of 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (0.5 M, pH 6.2, 1 mL) was added. After the addition of a recombinant FKP from *B. fragilis* strain NCTC9343 (Yi et al. 2009) (2.3 mg, FKP is a bifunctional L-fucokinase/GDP-fucose pyrophosphorylase which catalyzes the synthesis of GDP-fucose from L-fucose, ATP and GTP via a fucose-1-phosphate (Fuc-1-P) intermediate) and MBP–HhFT1–His$_6$ (0.9 mg), water was added to bring the volume of the reaction mixture to 10 mL. The reaction was carried out by incubating the solution in an incubator shaker for 72 h at 37 °C. The supplements of additional 0.5 equivalents of ATP (18 mg) and GTP (17 mg) were added periodically in every 12 h (2.5 equivalent totals). The reaction was quenched by adding cold ethanol (10 mL) and was then centrifuged to remove precipitates. A BioGel P-2 filtration and a silica gel column (EtOAc:MeOH:H_2O, 7:2:1) were used to purify the product to afford Galβ1-4(Fucα1-3)GlcNAcβ-ProN$_3$ (25 mg, 63 %).

pH profile by HPLC

Assays were performed in a total volume of 10 μL in a buffer (200 mM) with pH varying from 5.0 to 11.0 containing GDP-fucose (12 mM) with LacNAcβPro2AA (10 mM) or GDP-fucose (10 mM) with Neu5Acα2-3LacNAcβPro2AA (5 mM) and the recombinant enzyme (9.0 μg). Reactions were allowed to proceed for 60 min (when LacNAcβPro2AA was used as an acceptor) or 30 min (when Neu5Acα2-3LacNAcβPro2AA was used as an acceptor) at 37°C before being quenched by adding ice-cold 12% acetonitrile (1990 μL) to make 200-fold dilutions. The samples were then kept on ice until aliquots of 5 μL were injected and analyzed by a Shimadzu LC-2010A system equipped with a membrane online degasser, a temperature control unit and a fluorescence detector. A reverse-phase Premier C18 column (250 × 4.6 mm i.d., 5 μm particle size, Shimadzu) protected with a C18 guard column cartridge was used. The mobile phase was 18% acetonitrile when LacNAcβ-Pro2AA was used as an acceptor or 6.9% acetonitrile with 6.9% methanol when Neu5Acα2-3LacNAcβPro2AA was used as an acceptor. Fluorescence of the products LacNAcProβ2AA and Neu5Acα2-3LacNAcβPro2AA and the acceptor substrates were detected by excitation at 315 nm and emission at 400 nm. All assays were carried out in duplicate.

Effects of metal Ions, EDTA and DTT

EDTA (5 and 10 mM), different concentrations (5, 10, 20, and 50 mM) of $MgCl_2$ or $MnCl_2$, and various concentrations of DTT (0.2, 1, and 5 mM) with 20 mM $MgCl_2$ were used in HEPE buffer (pH 7.0, 100 mM) to analyze their effects on the fucosyltransferase activity of the recombinant enzyme (9.0 μg). A reaction without EDTA, DTT and metal ions was used as a control. The concentrations of the substrates and other reaction conditions were the same as described above for the pH profile assays.

Kinetics by HPLC assay

The assays were carried out in a total volume of 10 μL in HEPES buffer (100 mM, pH 7.0) containing $MgCl_2$ (20 mM), GDP-fucose, acceptor substrate (LacNAcβPro2AA or Neu5Acα2-3LacNAcβPro2AA) and the recombinant protein (9.0 μg). Reactions were allowed to proceed for 20 min at 37°C. Apparent kinetic parameters were obtained by varying the GDP-fucose concentration from 2.0 to 50.0 mM (2.0, 3.0, 5.0, 10.0, 20.0, and 50.0 mM) and a fixed concentration of LacNAcβPro2AA or Neu5Acα2-3LacNAcβPro2AA (5 mM); or a fixed concentration of GDP-fucose (10 mM) and varied concentrations of LacNAcβPro2AA or Neu5Acα2-3LacNAcβPro2AA (1, 1.5, 2.5, 5.0, 10.0, and 25.0 mM). Apparent kinetic parameters were obtained by fitting the data (the average values of duplicate assay results) into the Michaelis–Menten equation using Grafit 5.0.

pH profile of GDP-fucose hydrolysis by capillary electrophoresis assays

Assays were performed in a total volume of 10 μL in a buffer (200 mM) with pH varying from 6.0 to 10.0 containing GDP-fucose (10 mM), $MgCl_2$ (20 mM) and the recombinant enzyme (9.0 μg). Reactions were allowed to proceed for 30 min at 37°C before being quenched by adding ice-cold water (90 μL) to make 10-fold dilutions. The samples were then kept on ice until aliquots of 6 μL were withdrawn and analyzed by a Beckman P/ACE MDQ capillary electrophoresis system (60 cm × 75 μm i.d.) with a PDA detector. The ratio of the absorbance for GDP-fucose and GDP at 254 nm was determined at different concentrations (2.5, 5 and 10 mM). All assays were carried out in duplicate.

Kinetics of GDP-fucose hydrolysis by capillary electrophoresis assays

The enzymatic assays were carried out in a total volume of 10 μL in HEPES buffer (100 mM, pH 7.0) containing $MgCl_2$ (20 mM), GDP-fucose and the recombinant protein (9.0 μg). Reactions were allowed to proceed for 20 min at 37°C. Appar-

ent kinetic parameters were obtained by varying the GDP-fucose concentration from 2.0 to 50.0 mM (2.0, 3.0, 5.0, 10.0, 20.0 and 50.0 mM). Apparent kinetic parameters were obtained by fitting the data (the average values of duplicate assay results) into the Michaelis–Menten equation using Grafit 5.0.

Fucosidase activity studies

Assays were carried out in a total volume of 10 μL in HEPES buffer (100 mM, pH 7.0) containing $MgCl_2$ (20 mM), Le^x-β$ProN_3$ (10 mM) and various amounts of the recombinant protein (9.0, 18.0 or 27.0 μg). After 1 h reaction, the crude reaction mixture was analyzed by TLC. In addition, both Le^xβMU and Neu5Acα2-3Le^xβMU (5 mM) were used as substrates to test the fucosidase activity of the recombinant enzyme in a buffer (200 mM) containing $MgCl_2$ (20 mM) with pH varying from 5.0 to 8.0.

Product inhibition studies using GDP or Le^xβ$ProN_3$ as an inhibitor

Different concentrations (1, 5 and 10 mM) of Le^xβ$ProN_3$ or GDP (10 mM) were added to the reaction mixture to analyze its effect on the fucosyltransferase activity of the recombinant enzyme. Reaction without Le^xβ$ProN_3$ or GDP in HEPES buffer (pH 7.0, 100 mM) containing $MgCl_2$ (20 mM), LacNAcβPro2AA (5 mM), GDP-Fucose (10 mM) and the recombinant enzyme (9.0 μg) was used as a control.

Funding

This work was supported by the Alfred P. Sloan Research Fellowship and Camille Dreyfus Teacher-Scholarship (to X. Chen) and NIH R01HD061935 (to P.G. Wang).

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Conflict of interest statement

None declared.

Abbreviations

ATP, adenosine 5′-triphosphate; BCA, bicinchoninic acid; CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; CAPSO, *N*-cyclohexyl-2-hydroxyl-3-aminopropanesulfonic acid; CAZy, Carbohydrate Active Enzyme; DTT, dithiothreitol; FKP, L-fucokinase/GDP-fucose pyrophosphorylase; FT, fucosyltransferase; Fuc, fucose; Gal, galactose; GDP-Fuc, guanosine-5′-diphosphate L-fucose; GlcNAc, *N*-acetylglucosamine; GT, glycosyltransferase; GTP, guanosine-5′-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HhFT1, *Helicobacter hepaticus* α1-3-fucosyltransferase; HhFT2, *H. hepaticus* fucosyltransferase; HPLC, high-performance liquid chromatography; LacNAc, *N*-acetyllactosamine; Le^x, Lewis x; LPS, lipopolysaccharides; MBP, maltose binding protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; Neu5Ac, *N*-acetylneuraminic acid; NMR, nuclear magnetic resonance; PCR, polymerase chain reactions; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SLe^x, sialyl Lewis x; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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