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Fucosylation of chitooligosaccharides by human α1,6-fucosyltransferase requires a nonreducing terminal chitotriose unit as a minimal structure

Hideyuki Ihara[2], Shinya Hanashima[3], Takahiro Okada[2,6], Ritsu Ito[2], Yoshiki Yamaguchi[3], Naoyuki Taniguchi[4,5], and Yoshitaka Ikeda[1,2,6]

[2]Division of Molecular Cell Biology, Department of Biomolecular Sciences, Saga University Faculty of Medicine, 5-1-1 Nabeshima, Saga 849-8501, Japan, [3]Structural Glycobiology Team, Systems Glycobiology Research Group, Chemical Biology Department, RIKEN Advanced Science Institute, 2-1 Hirosawa Wako, Saitama 351-0198, Japan, [4]Department of Disease Glycomics, The Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan, [5]Disease Glycomics Team, Systems Glycobiology Research Group, Chemical Biology Department, RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan, and [6]Core Research for Evolutionary Science and Technology, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

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FUT8, a eukaryotic α1,6-fucosyltransferase, catalyzes the transfer of a fucosyl residue from guanine nucleotide diphosphate-β-L-fucose to the innermost GlcNAc of an asparagine-linked oligosaccharide (*N*-glycan). The catalytic domain of FUT8 is structurally similar to that of NodZ, a bacterial α1,6-fucosyltransferase, which acts on a chitooligosaccharide in the synthesis of Nod factor. While the substrate specificities for the nucleotide sugar and the *N*-glycan have been determined, it is not known whether FUT8 is able to fucosylate other sugar chains such as chitooligosaccharides. The present study was conducted to investigate the action of FUT8 on chitooligosaccharides that are not generally thought to be a substrate in mammals, and the results indicate that FUT8 is able to fucosylate such structures in a manner comparable to NodZ. Surprisingly, structural analyses of the fucosylated products by high performance liquid chromatography, mass spectrometry and nuclear magnetic resonance indicated that FUT8 does not utilize the reducing terminal GlcNAc for fucose transfer but shows a preference for the third GlcNAc residue from the nonreducing terminus of the acceptor. These findings suggest that FUT8 catalyzes the fucosylation of chitooligosaccharide analogous to NodZ, but that a nonreducing terminal chitotriose structure is required for the reaction. The substrate recognition by which FUT8 selects the position to fucosylate might be distinct from that for NodZ and could be due to structural factor requirements which are inherent in FUT8.

Keywords: chitooligosaccharide/fucosylation/fucosyltransferase/FUT8/NodZ

Introduction

FUT8, a eukaryotic α1,6-fucosyltransferase, catalyzes the transfer of a fucosyl residue from guanine nucleotide diphosphate (GDP)-β-L-fucose to the reducing terminal GlcNAc of asparagine-linked oligosaccharides (*N*-glycan) (Wilson et al. 1976) (Figure 1). This fucose moiety is referred to as a core fucose residue, and is widely distributed in nature, from insects to animals (Oriol et al. 1999; Staudacher et al. 1999). As previously reported, it has been suggested that various biological events are regulated via the core fucosylation of various glycoproteins (Miyoshi et al. 1999; Taniguchi et al. 2006). The enzymatic properties of FUT8 have been extensively investigated, especially in terms of substrate specificities and the reaction mechanism (Longmore and Schachter 1982; Voynow et al. 1991; Shao et al. 1994; Kaminska et al. 1998; Staudacher and Marz 1998; Paschinger et al. 2005; Ihara et al. 2006).

In the fucose transfer reaction, FUT8 requires the presence of a β1,2-GlcNAc residue linked to the α1,3-mannose arm in the tri-mannose core structure of an *N*-glycan (Longmore and Schachter 1982; Voynow et al. 1991; Shao et al. 1994; Kaminska et al. 1998; Paschinger et al. 2005) and acts on only the β-anomer of the reducing terminal GlcNAc which is linked to the asparagine residue in the *N*-glycosylation site (Voynow et al. 1991). On the other hand, some modifications of the oligosaccharides function as inhibitory factors for FUT8 reactions: the galactosylation of the β1,2-GlcNAc residue linked to the α1,3-mannose arm (Voynow et al. 1991), addition of the bisecting GlcNAc that is catalyzed by β1,4-*N*-acetylglucosaminylytransferase (GnT-III) (Longmore and Schachter 1982) and the α1,3-fucosylation of the innermost GlcNAc residue, as observed in insects (Staudacher and Marz 1998; Paschinger et al. 2005). Concerning the substrate specificity for the donor, our previous study indicated that FUT8 strongly recognizes both the base portion and the diphosphoryl group of the donor nucleotide sugar (Ihara et al. 2006).

We recently reported on the crystal structure of human FUT8, and the results showed that the structure of FUT8 includes a catalytic domain and two additional domains, an N-terminal coiled-coil structure and a C-terminal SH3 domain (Ihara et

[1]To whom correspondence should be addressed: Tel: +81-952-34-2190; Fax: +81-952-34-2189; e-mail: yikeda@med.saga-u.ac.jp

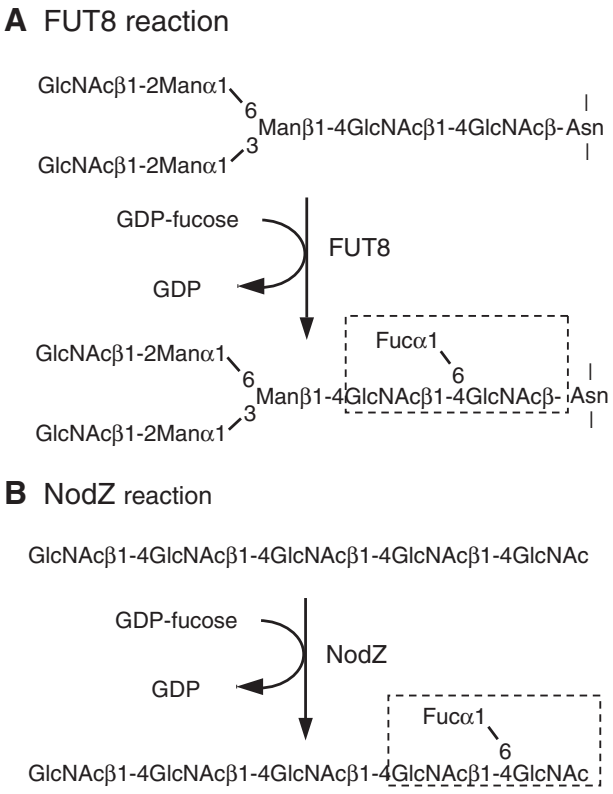

Fig. 1. Reactions catalyzed by FUT8 (**A**) and NodZ (**B**). FUT8 transfers a fucose residue from GDP-β-L-fucose to the innermost GlcNAc of *N*-glycan via an α1,6-linkage. NodZ also transfers fucose to chitooligosaccharide via a reaction similar to FUT8. Dashed boxes denote the common structure of products synthesized by enzymes.

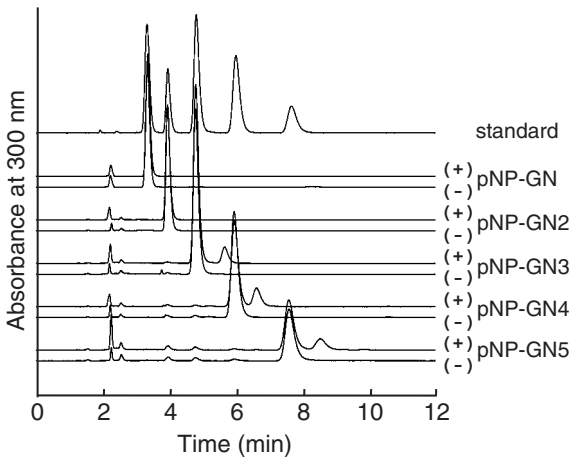

Fig. 2. Fucosylation of pNP-labeled chitooligosaccharide by FUT8. FUT8 reactions were carried out with various pNP-labeled chitooligosaccharides in the presence (+) or absence (−) of GDP-β-L-fucose as donor substrate. pNP-GN2, pNP-GN3, pNP-GN4 and pNP-GN5 represent pNP-chitobiose, pNP-chitotriose, pNP-chitotetraose and pNP-chitopentaose, respectively. Nonfucosylated and fucosylated chitooligosaccharides were separated by normal phase HPLC as described under Materials and methods. The labeled oligosaccharides were detected by UV absorbance at 300 nm for the pNP group. The profile for STD indicates the elution of pNP-GN2, pNP-GN3, pNP-GN4 and pNP-GN5 as a standard sample.

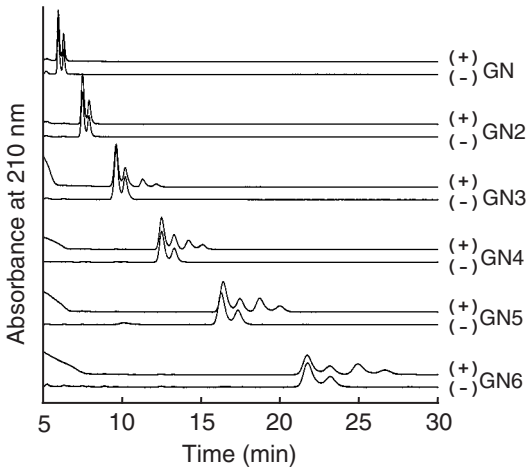

Fig. 3. Fucosylation of chitooligosaccharide by FUT8. FUT8 reactions with 1 mM of various chitooligosaccharides were carried out with (+) or without (−) GDP-β-L-fucose as donor substrate. GN, GN2, GN3, GN4, GN5 and GN6 denote *N*-acetylglucosamine, chitobiose, chitotriose, chitotetraose, chitopentaose and chitohexaose, respectively. The reaction products were analyzed by normal phase HPLC. Elution was monitored by a UV detector at 210 nm. The detailed procedures are described under Materials and methods.

al. 2007). In addition, it was found that the enzyme can be structurally classified as members of the GT-B group of glycosyltransferases (Coutinho et al. 2003; Qasba et al. 2005). The structure of bacterial α1,6-fucosyltransferase, referred to as NodZ, has also been solved (Brzezinski et al. 2007), and a structural comparison between FUT8 and NodZ showed that the catalytic domains of these two enzymes are very similar. NodZ plays a role in the synthesis of the Nod factor, which is involved in the nodulation of legumes root for nitrogen-fixing, and is known to catalyze the α1,6-fucosylation of lipo-chitooligosaccharide and variations thereof, including chitooligosaccharides (Quesada-Vincens et al. 1997). Both the mammalian and bacterial fucosyltransferases are classified into

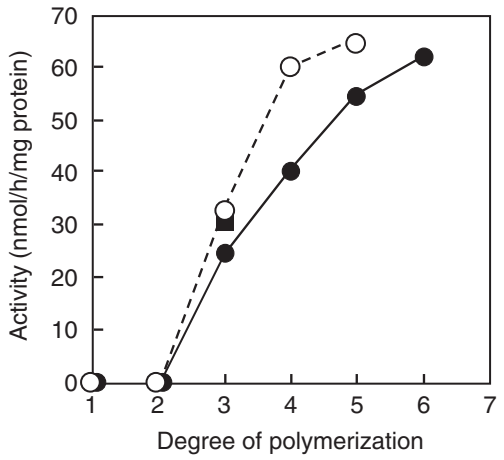

Fig. 4. Correlation between the activity and degree of polymerization of chitooligosaccharide in the fucosylation by FUT8. The closed circles indicate nonlabeled chitooligosaccharides series. The open circles indicate pNP-labeled chitooligosaccharides series. The closed square represents 4MU-labeled chitotriose.

the GT23 family of Carbohydrate-Active enZYmes (Campbell et al. 1997) and share GDP-β-L-fucose as the donor substrate (Figure 1). Although the acceptor substrates are different, a "common" chitobiose unit is contained in the reducing terminals of both substrates. Thus, in addition to the structural similarities, it is reasonable to expect that these mammalian and bacterial enzymes are functionally similar in their other enzymatic properties. Furthermore, a comparison of the primary structures of FUT8, NodZ, α1,2- and protein *O*-fucosyltransferases indicates that all contain three small, highly conserved regions (Breton et al. 1998; Oriol et al. 1999; Takahashi et al. 2000; Chazalet et al. 2001; Martinez-Duncker et al. 2003), and the spatial arrangement of these regions is essentially the same in the steric structures of FUT8 and NodZ (Brzezinski et al. 2007; Ihara et al. 2007).

Chitooligosaccharides are precursors of the Nod factor and serve as acceptor substrates for NodZ, while an *N*-glycan is an acceptor substrate for FUT8. However, it was reported that NodZ can utilize *N*-glycan as an acceptor substrate and actually fucosylate it to a lesser extent, compared to FUT8 (Quinto et al. 1997). This observation suggests that the bacterial enzyme displays a broad specificity toward the acceptor, and it appears that the enzyme does not strictly recognize the substrate. On the other hand, an earlier study showed that chitobiose and chitotriose do not serve as active substrates for FUT8, and it seemed likely that FUT8 acts exclusively on *N*-glycans (Voynow et al. 1991). However, because chitooligosaccharides that are sufficiently active substrates for NodZ have not been examined for fucosylation by FUT8, it is not known whether the specificity of FUT8 toward the acceptor is broad or not, as reported for NodZ. In this study, we examined and characterized the reactions of FUT8 with various chitooligosaccharides as acceptor substrates. As for the results, the fucosylation of the chitooligosaccharide acceptors

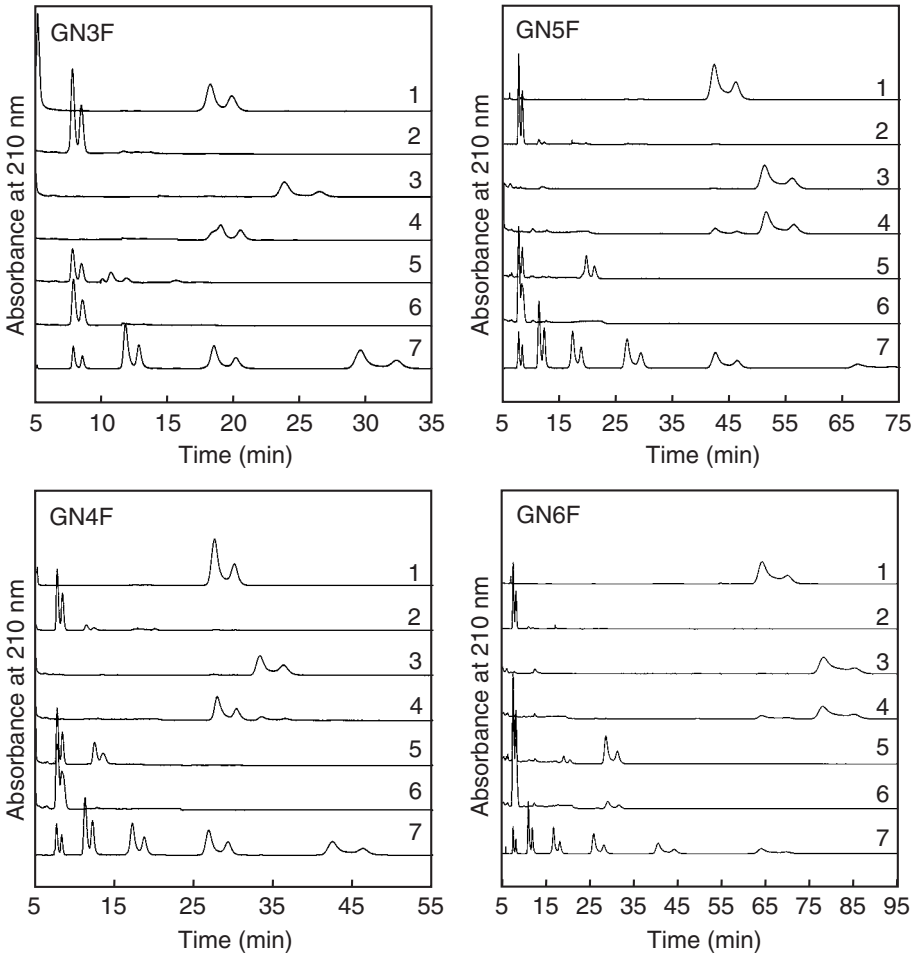

Fig. 5. Structural analyses of fucosylated chitooligosaccharides obtained by glycosidase digestion. The fucosylated chitooligosaccharides by FUT8 were digested by β-*N*-acetylhexosaminidase and α-fucosidase, and analyzed by normal phase HPLC analysis. The detailed procedures are described in Materials and methods. Numbers for chromatographs in each panel represent 1, nonfucosylated chitooligosaccharide substrate; 2, β-*N*-acetylhexosaminidase digestion of the nonfucosylated chitooligosaccharide; 3, fucosylated chitooligosaccharide product; 4, fucosidase digestion of the fucosylated chitooligosaccharide; 5, β-*N*-acetylhexosaminidase digestion of the fucosylated chitooligosaccharide; 6, β-*N*-acetylhexosaminidase and fucosidase digestion of the fucosylated chitooligosaccharide; 7, standard oligosaccharides.

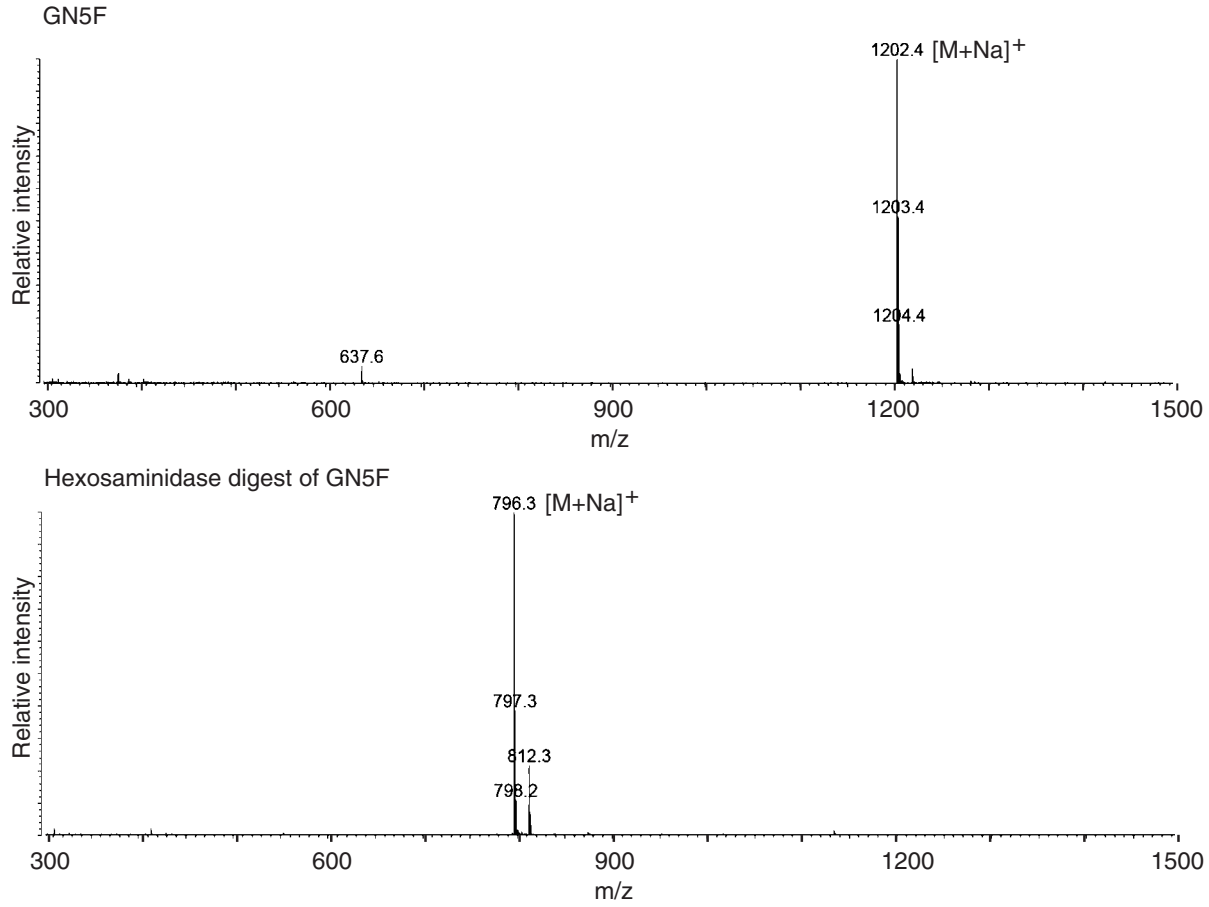

Fig. 6. MALDI-TOF MS spectra of GN5F and the β-*N*-acetylhexosaminidase digest. GN5F; calculated as 1202.46 $[C_{46}H_{77}N_5O_{30}+Na]^{+}$, found 1202.5. The β-*N*-acetylhexosaminidase digest; calculated as GN3F 796.30 $[C_{30}H_{51}N_2O_{20}+Na]^{+}$, found 796.3.

was observed, and it was found that FUT8 exhibits a "NodZ-like" fucosylating activity. However, detailed analyses showed certain difference in the nature by which the acceptors are fucosylated. Furthermore, our findings would also provide new insights into how FUT8 fucosylates oligosaccharide substrates at a specific position.

Table 1. ^{1}H- and ^{13}C-chemical shifts of GN5F

^{1}H, ^{13}C chemical shifts (ppm)						
Residue	1	2	3	4	5	6
Fucose	102.5 4.87	72.3 3.88	71.0 3.77	74.7 3.79	69.7 4.04	18.2 1.22
GlcNAc (A)	93.2 (α), 97.6 (β) 5.18 (α), 4.68 (β)	56.4 (α), 58.9 (β) 3.87 (α), 3.68 (β)	72.0 (α), 74.9 (β) 3.87 (α), 3.70 (β)	82.3 3.62	72.6 (α), 77.4 (β) 3.87 (α), 3.53 (β)	62.8 3.65, 3.83
GlcNAc (B)	104.3 4.57	57.9 3.77	74.9 3.70	82.3 3.62	77.4 3.53	62.8 3.65, 3.83
GlcNAc (C)	104.3 4.57	57.9 3.77	76.1 3.69	81.2 3.77	77.4 3.53	69.7 3.69, 3.91
GlcNAc (D)	104.3 4.57	57.9 3.77	74.9 3.70	82.3 3.62	77.4 3.53	62.8 3.65, 3.83
GlcNAc (E)	104.3 4.57	57.9 3.77	72.5 3.47	76.2 3.56	78.7 3.48	63.3 3.74, 3.91

Chemical shifts (ppm) of ^{1}H- and ^{13}C-NMR of GN5F were performed in D_2O at 25°C, and the chemical shifts were calibrated with DSS (0 ppm). The structure and residue notation of oligosaccharide analyzed are described as GlcNAc(E)β1-4GlcNAc(D)β1-4 (Fucα1-6)GlcNAc(C)β1-4GlcNAc(B)β1-4GlcNAc(A).

Table 2. ^{1}H- and ^{13}C-chemical shifts of GN5

^{1}H, ^{13}C chemical shifts (ppm)						
Residue	1	2	3	4	5	6
GlcNAc (A)	93.3 (α), 97.6 (β) 5.18 (α), 4.69 (β)	56.5 (α), 59.0 (β) 3.87 (α), 3.68 (β)	72.0 (α), 74.9 (β) 3.87 (α), 3.71 (β)	81.8 3.64	72.9 (α), 77.4 (β) 3.87 (α), 3.54 (β)	62.8 3.65, 3.84
GlcNAc (B)	104.1 4.57	57.9 3.76	74.9 3.71	81.8 3.64	77.4 3.54	62.8 3.65, 3.84
GlcNAc (C)	104.1 4.57	57.9 3.76	74.9 3.71	81.8 3.64	77.4 3.54	62.8 3.65, 3.84
GlcNAc (D)	104.1 4.57	57.9 3.76	74.9 3.71	81.8 3.64	77.4 3.54	62.8 3.65, 3.84
GlcNAc (E)	104.1 4.57	57.9 3.76	72.5 3.47	76.3 3.56	78.7 3.48	63.4 3.74, 3.91

Chemical shifts (ppm) of ^{1}H- and ^{13}C-NMR of GN5 were performed in D_2O at 25°C, and the chemical shifts were calibrated with DSS (0 ppm). The structure and residue notation of oligosaccharide analyzed are described as GlcNAc(E)β1-4GlcNAc(D)β1-4GlcNAc(C)β1-4GlcNAc(B)β1-4GlcNAc(A).

Table 3. ^{1}H- and ^{13}C-chemical shifts of β-*N*-acetylhexosaminidase digest of GN5F

^{1}H, ^{13}C chemical shifts (ppm)						
Residue	1	2	3	4	5	6
Fucose	102.5 4.90	72.2 3.87	71.2 3.74	74.6 3.79	69.7 4.08	18.1 1.22
GlcNAc (A)	93.2 (α), 97.7 (β) 5.18 (α), 4.68 (β)	56.5 (α), 58.8 (β) 3.87 (α), 3.68 (β)	72.2 (α), 75.2 (β) 3.87 (α), 3.69 (β)	82.4 3.62	72.2 (α), 76.2 (β) 3.87 (α), 3.57 (β)	62.8 3.65, 3.77 (α) 3.65, 3.82 (β)
GlcNAc (B)	103.9 4.60	58.1 3.78	75.2 3.69	82.4 3.62	77.0 3.59	62.8 3.65, 3.82
GlcNAc (C)	104.6 4.58	58.1 3.78	72.7 3.54	77.6 3.66	77.4 3.50	70.2 3.75, 4.00

Chemical shifts (ppm) of ^{1}H- and ^{13}C-NMR of β-*N*-acetylhexosaminidase digest of GN5F were performed in D_2O at 25°C, and the chemical shifts were calibrated with DSS (0 ppm). The structure and residue notation of oligosaccharide analyzed are described as Fucα1-6GlcNAc(C)β1-4GlcNAc(B)β1-4GlcNAc(A).

Table 4. ^{1}H- and ^{13}C-chemical shifts of GN3

^{1}H, ^{13}C chemical shifts (ppm)						
Residue	1	2	3	4	5	6
GlcNAc (A)	93.2 (α), 97.7 (β) 5.18 (α), 4.69 (β)	56.4 (α), 58.9 (β) 3.87 (α), 3.68 (β)	72.0 (α), 75.1 (β) 3.87 (α), 3.69 (β)	82.0 3.64	72.9 (α), 77.3 (β) 3.87 (α), 3.78 (β)	62.8 3.66, 3.78 (α) 3.66, 3.84 (β)
GlcNAc (B)	104.1 4.58	58.0 3.77	75.0 3.72	82.0 3.64	77.3 3.56	62.8 3.66, 3.84
GlcNAc (C)	104.1 4.58	58.4 3.74	72.6 3.48	76.3 3.56	78.7 3.49	63.3 3.75, 3.91

Chemical shifts (ppm) of ^{1}H- and ^{13}C-NMR of β-*N*-acetylhexosaminidase digest of GN3 were performed in D_2O at 25°C, and the chemical shifts were calibrated with DSS (0 ppm). The structure and residue notation of oligosaccharide analyzed are described as GlcNAc(C)β1-4GlcNAc(B)β1-4GlcNAc(A).

Results

In general, when the enzyme activities of glycosyltransferases that act on *N*-glycans are assayed, oligosaccharides labeled via reductive amination are often used for sensitive detection. For example, it is well known that 2-aminopyridine (Hase et al. 1981), 8-aminonaphthalene-1,3,6-trisulfonic acid (Klockow et al. 1995) and a hydrazide-derivative of naphthalene (Muramoto et al. 1994; Leteux et al. 1998) are very useful for quantitative analyses involving high performance liquid chromatography (HPLC) and electrophoresis. However, these methods for labeling of *N*-glycans cannot be used for the assay of FUT8 because the enzyme requires that the innermost GlcNAc exists in a closed ring form and cannot act on an opened form. In fact, most of the oligosaccharides used for the FUT8 assay are an asparagine-linked form in which the carboxyl or the amino group of the aglycon is modified by a chromophore or fluorophore (Uozumi, Teshima et al. 1996; Roitinger et al. 1998; Martinez-Duncker et al. 2004; Ihara et al. 2006). Thus, *O*-*p*-nitrophenyl (pNP) and *O*-4-methylumbel-

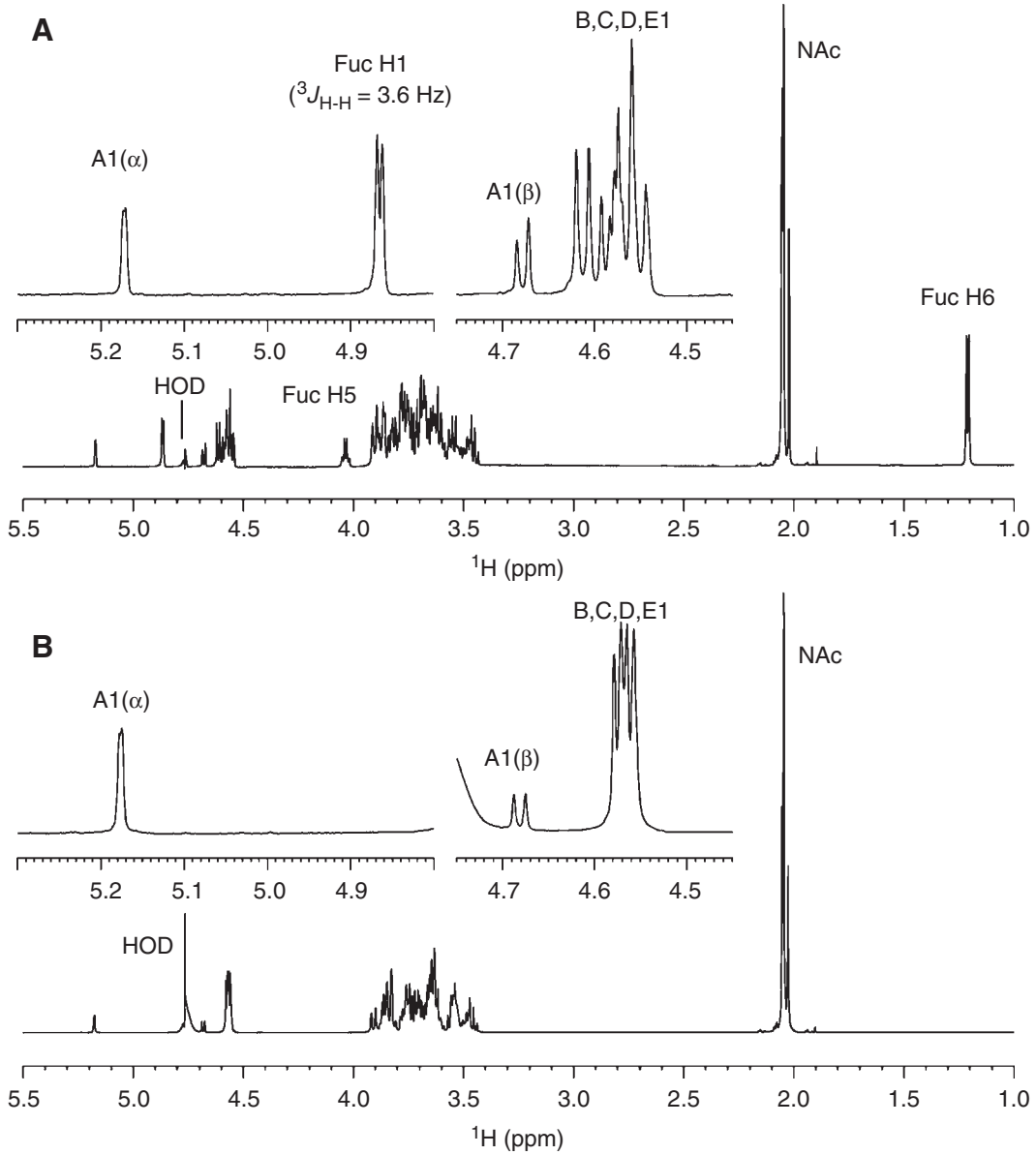

Fig. 7. NMR analyses of GN5F. ^{1}H-NMR spectra of GN5F and GN5 are shown in (**A**) and (**B**), respectively. The spectra were measured using a 600 MHz spectrometer and the chemical shifts were calibrated using the DSS singlet adjusted at 0.00 ppm. H1, H5 and H6 signals assigned to Fuc residue are indicated in the spectrum (**A**). The anomeric configuration of the fucose residue was confirmed as the α-form because the value of the $^3J_{H1-H2}$ coupling constant (3.6 Hz) is the typical value for a gauche configuration. The anomeric area of the ^{1}H-^{13}C HSQC spectra of GN5F and GN5 is shown in (**C**) and (**D**), respectively. The anomeric ^{1}H-^{13}C correlation signal of Fuc residue is found in (**C**). The nonanomeric area of GN5F and GN5 is indicated in (**E**) and (**F**), respectively. HSQC signals of Fuc 2-, 3-, 4- and 5-positions are found in (**E**). The detailed procedures are described in Materials and methods. The structure and residue notations for the oligosaccharides analyzed are shown in Tables 1 and 2.

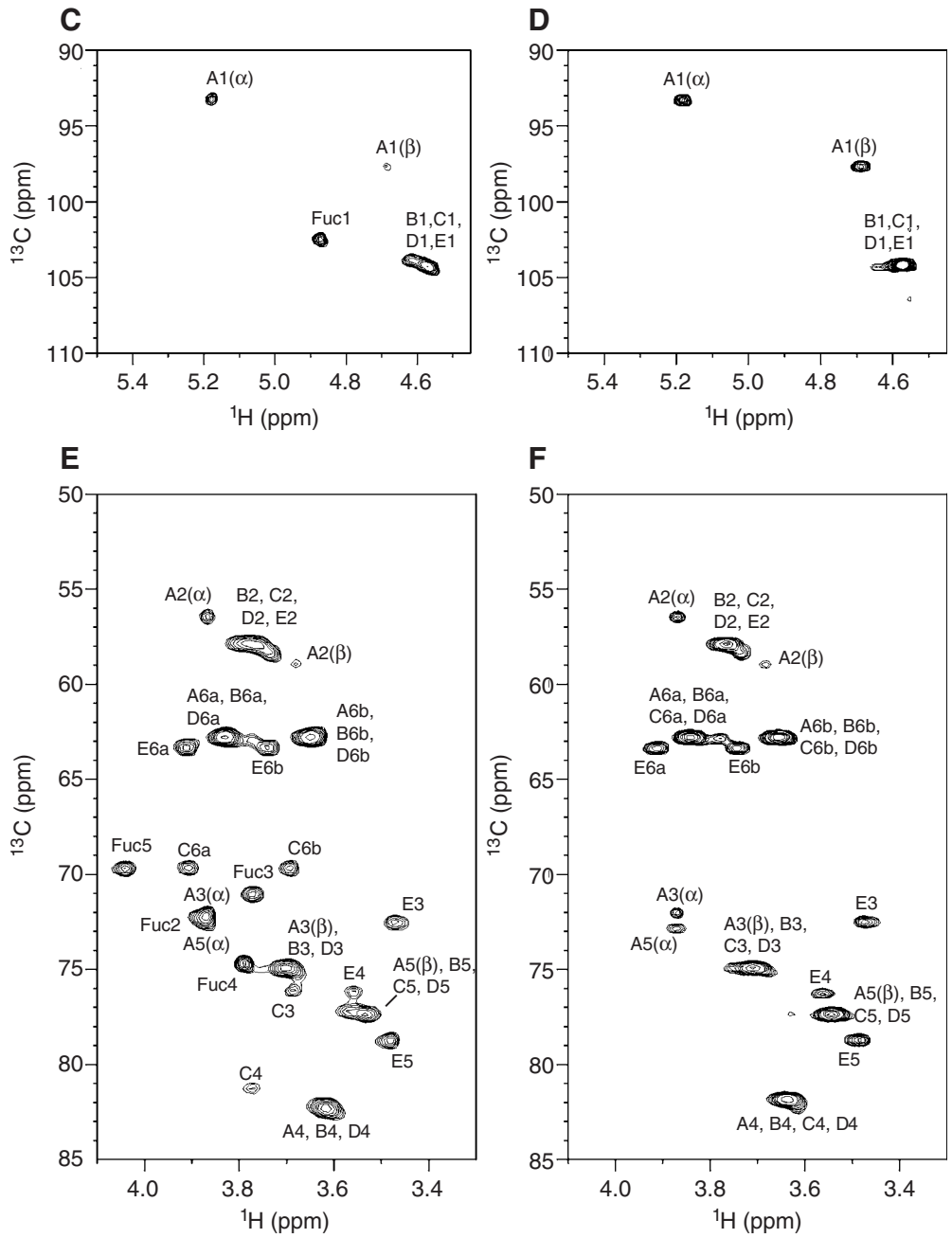

Fig. 7. (*continued*).

liferyl (4MU) forms of various chitooligosaccharides, all of which are commercially available, were examined for use in the assay for the fucosylation by FUT8.

The labeled chitooligosaccharides were incubated with purified recombinant FUT8 in the presence of GDP-β-L-fucose, and the products of the reaction were analyzed by normal phase HPLC using an ultraviolet (UV) or fluorescent detector. As shown in Figure 2, shifts in the peaks were observed, except for derivatives of GlcNAc and chitobiose, and the shifted peaks were not produced in the absence of the donor. No peak shift was found when the enzyme was omitted from the reaction (data not shown). Because fucose is a deoxy sugar, an oligosaccharide containing a fucose residue is not retained as much as a GlcNAc in normal phase HPLC. Therefore, peaks corresponding to fucosylated chitooligosaccharides wedged between those of GlcNAc polymers as the substrates in the elution profiles. These properties allowed separation of the substrates and products. The results indicate that FUT8 is

clearly able to catalyze the fucosylation of chitooligosaccharides that contain three or more GlcNAc, in addition to *N*-glycans. As shown by the HPLC analyses in Figure 2, only a single shifted peak was observed in the elution profiles of the chitooligosaccharides that were fucosylated by FUT8, which suggests that the oligosaccharides are mono-fucosylated regardless of the degree of polymerization of the substrate.

Although the findings show that FUT8 is able to glycosylate pNP or other derivatives of chitooligosaccharides as well as *N*-glycans, the actual substrates for NodZ contain a free reducing terminal. Thus, while labeling of the oligosaccharides with a chromophore or fluorophore is useful for sensitive detection, but the possibility that such an aglycon might play a role in assisting the enzymatic reaction cannot be excluded. Hence, in order to more appropriately evaluate the activity of FUT8 on chitooligosaccharides, unlabeled substrates were examined for the fucosylation by FUT8. In these substrates, the reducing group is present as both α and β anomers, which are in equilibrium and this could complicate the assay as compared to the use of a labeled substrate.

As shown in Figure 3, when FUT8 was incubated with GDP-β-L-fucose and the chitooligosaccharides as acceptors, peaks corresponding to fucosylated oligosaccharides were found in the cases of chitotriose (GN3), chitotetraose (GN4), chitopentaose (GN5) and chitohexaose (GN6). In these analyses, using the normal phase mode, each sugar chain, including the fucosylated one, was successfully detected as a combination of two separate peaks that are based on anomeric configuration,

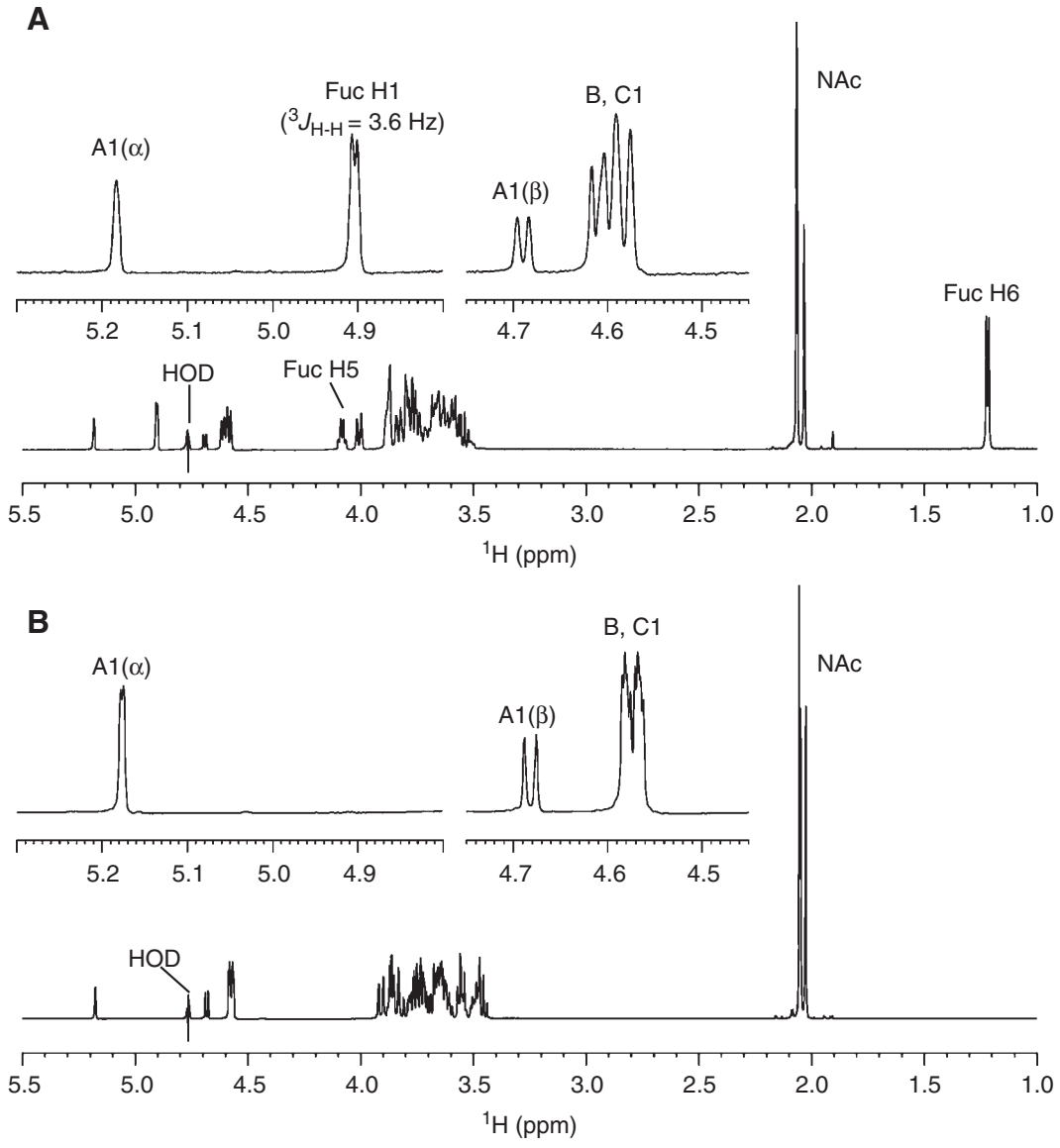

Fig. 8. NMR analysis of the β-*N*-acetylhexosaminidase digest of GN5F. ^{1}H-NMR spectra of the β-*N*-acetylhexosaminidase digest (**A**) are shown as well as a standard sugar, GN3, (**B**) to compare. The H1, H5 and H6 signals assigned to Fuc residue are indicated in the spectrum (**A**). The $^3J_{H1-H2}$ coupling constant of the Fuc residue was 3.6 Hz, which was identical to the corresponding value of fucose in GN5F. The anomeric area of the ^{1}H-^{13}C HSQC spectra is shown in (**C**) and (**D**), respectively. The nonanomeric area of the digest and GN3 is indicated in (**E**) and (**F**), respectively. The HSQC signals assigned to Fuc residue are shown in (**C**) and (**E**). The detailed procedures are described in Materials and methods. The structure and residue notations of oligosaccharide analyzed are shown in Tables 3 and 4.

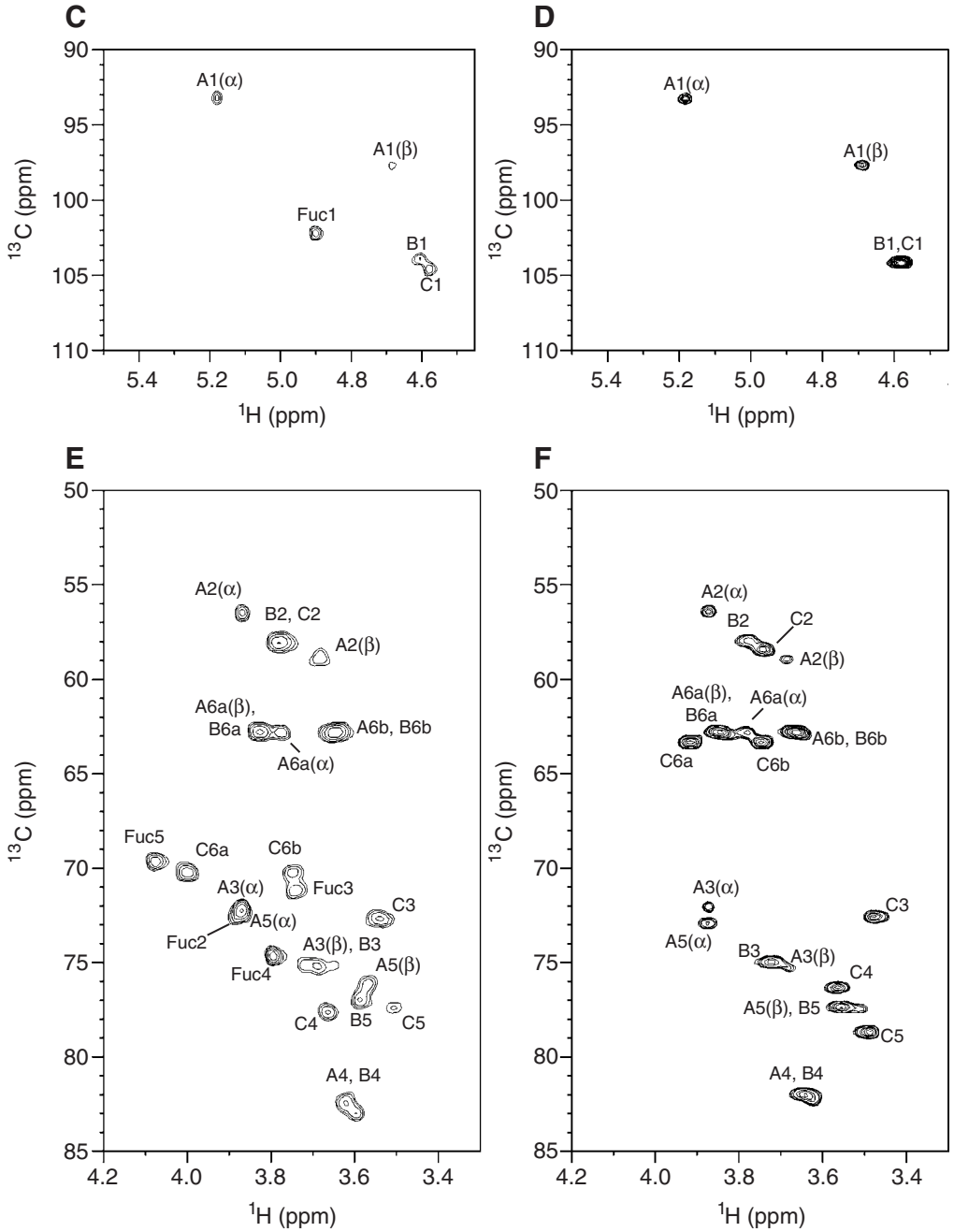

Fig. 8. (*continued*).

while the pNP and 4MU-labeled substrates were eluted as single peaks due to the fact that β-linkage at the reducing end is fixed. Thus, the analytical system used allowed the separation of the α- and β-anomer forms of chitooligosaccharides, as reported previously (Abdel-Banat et al. 2002; Kabir et al. 2006). The fucosylated products also showed only a pair of peaks that were based on two anomers as observed for the substrates, indicating that the substrates that underwent mono-fucosylation on the FUT8-conducted reactions. On the other hand, no product was detected when GlcNAc (GN) and chitobiose (GN2) were used as the acceptor (Figure 3). These results are essentially the same as those obtained for the labeled oligosaccharides and also suggest that the aglycon does not interfere with the reaction of FUT8 with chitooligosaccharides.

In order to evaluate the substrate preference of FUT8, the activities were assayed using various chitooligosaccharides or related substrates. No activity was observed in the cases of GlcNAc and chitobiose, either labeled or unlabeled (Figures 2 and 3). The specific activities for nonlabeled GN3, GN4,

GN5 and GN6 were 25, 40, 55 and 62 nmol/h/mg protein, respectively. The specific activities for pNP-GN3, pNP-GN4, pNP-GN5 and 4MU-GN3 were also 33, 60, 65 and 31 nmol/h/mg protein, respectively. These values are in the range of about 5–6% of the activity of FUT8 for *N*-glycans. As shown in Figure 4, a longer oligosaccharide is more active as the substrate, at least, in the range of the chain length examined. In addition, the activity appeared to reach a plateau in both the nonlabeled and pNP-labeled GNs, suggesting that an oligosaccharide with a length of 6–7 residues is sufficient for maximal activity. While the activity for nonlabeled GN3 was slightly lower than for the labeled samples, no significant difference was found between 4MU and pNP derivatives, suggesting that the β-anomeric configuration of the reducing terminal is important rather than the presence of an aglycon moiety. As shown by a comparison of the activities for nonlabeled and pNP-labeled acceptors, it appears that FUT8 prefers a fixed anomeric configuration to a free reducing terminal that is in anomeric equilibrium.

Previous reports showed that NodZ transfers a fucosyl residue to the reducing terminal GlcNAc of a chitooligosaccharide (Sanjuan et al. 1992; Bec-Ferté et al. 1994). In order to determine which GlcNAc residue in the chitooligosaccharide is fucosylated by FUT8, the reaction products were analyzed by exo-glycosidase digestions. The digestions were carried out using β-*N*-acetylhexosaminidase from jack bean and α-fucosidase from bovine kidney, and the digested samples were applied to normal phase HPLC analysis (Figure 5). When the samples were treated with fucosidase, the peaks in the elution profiles were shifted to the positions corresponding to the respective substrates that were used for the FUT8 reaction, as indicated by the fact that they were identical to nonfucosylated standards. These results confirm the α-fucosylation of GNs by FUT8, as expected from the aforementioned normal phase HPLC analyses. In the fucosidase digestion, while fucosylated chitotriose (GN3F) and tetraose (GN4F) were readily digested to nonfucosylated forms by α-fucosidase, fucosylated chitopentaose (GN5F) and hexaose (GN6F) were only slightly digested under the same conditions as were used for GN3F and GN4F, suggesting their fucosidase-resistant characteristics.

β-*N*-Acetylhexosaminidase treatment liberated free GlcNAc molecules and oligosaccharides shortened by two GlcNAc residues for all of the fucosylated chitooligosaccharides, whereas the nonfucosylated substrates were completely digested to free GlcNAc. It was most likely that the residual components that were resistant to further digestion by β-*N*-acetylhexosaminidase contain a fucose residue at the nonreducing terminal but not at the internal residue because the β-*N*-acetylhexosaminidase used in this study is an exo-type glycosidase and is unable to hydrolyze the substituted GlcNAc at the nonreducing terminal, for example, it is known that galactosyl GlcNAc inhibits the action of this enzyme (Li and Li 1970). In fact, treatment with a combination of β-*N*-acetylhexosaminidase and α-fucosidase led to complete degradation to monosaccharide components. This finding suggests that the third GlcNAc from the nonreducing terminal is fucosylated by FUT8 in chitooligosaccharides. In the case of GN6F, however, fucosylated chitotriose was observed as a minor component, along with to the major fucosylated chitotetraose, when digested by β-*N*-acetylhexosaminidase.

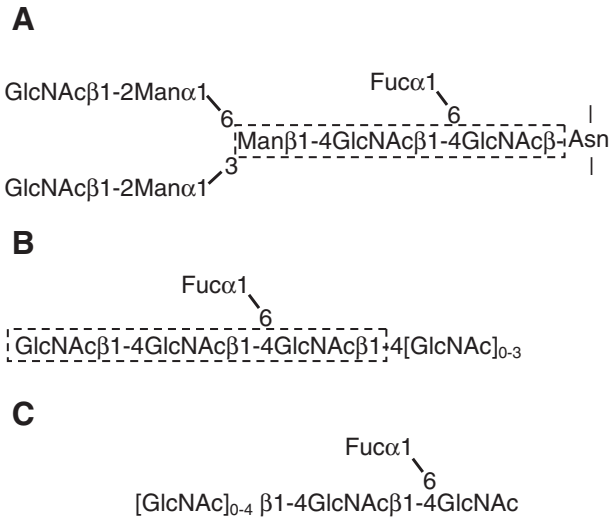

Fig. 9. Proposed structural requirement for an oligosaccharide substrate for fucosylation by FUT8. Structures of fucosylated forms of *N*-glycan and chitooligosaccharide are shown for FUT8 (**A** and **B**) and NodZ (**C**). Dashed boxes are the putative essential structures for the reaction of FUT8.

In order to further verify the fucosylation pattern obtained by glycosidase digestion and normal phase HPLC, nuclear magnetic resonance (NMR) and mass spectrometry (MS) experiments were performed. As shown in Figure 5, GN5F was prepared by the reaction of GN5 with FUT8 and purified by normal phase HPLC for NMR and MS analyses, and product obtained by β-*N*-acetylhexosaminidase digestion was also prepared and examined. Consistent with the chromatographic data (Figure 5), the MS analysis clearly showed that the difference in mass between these sugar chains is equivalent to two molecules of HexNAc (Figure 6), indicating that the β-*N*-acetylhexosaminidase treatment removed two GlcNAc residues from GN5F. The anomeric configuration and the position of the fucose residue in GN5F were determined by NMR. As summarized in Tables 1–4, the ^{1}H and ^{13}C-signals of GN5F and its β-*N*-acetylhexosaminidase digest as well as two unfucosylated sugars, GN5 and GN3, were assigned on the basis of 1D ^{1}H-NMR, 2D ^{1}H-^{1}H COSY (correlation spectroscopy), 2D ^{1}H-^{13}C HSQC (hetero-nuclear single quantum coherence) and 2D HSQC-TOCSY (total correlation spectroscopy) spectra. In the ^{1}H-NMR spectra of GN5F and the digest (Figures 7A and 8A), the observed $^{3}J_{H1-H2}$ coupling constant of the anomeric proton of fucose was 3.6 Hz, indicating that the fucose is attached to the chitooligosaccharides via an α-linkage. In the ^{1}H-^{13}C HSQC spectrum of GN5F, the ^{13}C-chemical shift of the C-6 for one GlcNAc residue was 69.7 ppm, while either 62.8 or 63.3 ppm for the other residues (Figure 7E, Table 1). On the other hand, in the spectrum of the unfucosylated form, GN5, all of the C-6 signals of GlcNAc were observed at either 62.8 or 63.4 ppm (Figure 7F, Table 2), but no signal was found at 69.7 ppm. The difference in the chemical shifts is consistent with the view that the fucose is attached to the C-6 position of a GlcNAc residue, although it was not possible to specify which GlcNAc residue is fucosylated, due to overlapping signals. In a comparison of the ^{1}H-^{13}C HSQC spectra of the β-*N*-acetylhexosaminidase-digested product and GN3, however, a similar difference was observed for the nonreducing terminal residues,

denoted by "GlcNAc (C)" (70.2 ppm vs 63.3 ppm, Figure 8E and F, Tables 3 and 4), thus indicating that the fucose residue is attached to the nonreducing terminal GlcNAc residue in the β-*N*-acetylhexosaminidase-digest of GN5F. This indicates that the fucose is present at the third GlcNAc residue, as indicated by the chromatographic analyses involving glycosidase digestion. These structural analyses support the conclusion that FUT8 fucosylates chitooligosaccharides via an α1,6-linkage at the third GlcNAc residue from the nonreducing terminal (Figure 9B).

Discussion

The fucosylation of chitooligosaccharides has been reported for α1,3- and α1,6-fucosylation (Sanjuan et al. 1992; Bec-Ferté et al. 1994; Olsthoorn et al. 1998; Natunen et al. 2001). The α1,3-fucosylation of chitooligosaccharides has been found in a natural product from rhizobium (Olsthoorn et al. 1998); however, the α1,3-fucosyltransferase responsible for the synthesis of the structure has not been identified in rhizobia. As indicated by the in vitro chemoenzymatic synthesis of a fucosylated oligosaccharide using a human enzyme, it is likely that the human α1,3-fucosyltransferase also has the ability to fucosylate chitooligosaccharides (Natunen et al. 2001). On the other hand, it is known that only NodZs from rhizobia catalyze the α1,6-fucosylation of chitooligosaccharide (Sanjuan et al. 1992; Bec-Ferté et al. 1994). This α1,6-fucosylation by NodZ occurs at the reducing terminal GlcNAc residue of the oligosaccharide, while the α1,3-fucosylation of GNs by rhizobial and human enzymes acts on internal GlcNAc residues. The findings herein show that human α1,6-fucosyltransferase has the ability to catalyze the synthesis of α1,6-fucosylated chitooligosaccharides as well as the core fucosylation of *N*-glycans, and furthermore that the α1,6-fucosylated chitooligosaccharide formed by the mammalian enzyme is distinct from that produced by the bacterial enzymes in terms of the position of fucosylation.

Interestingly, while NodZ fucosylates the reducing terminal GlcNAc (Figure 9), our findings indicated that FUT8 transfers a fucosyl unit to the third GlcNAc position from the nonreducing terminal of chitooligosaccharides, suggesting that the mechanism(s) by which the residue in the acceptor oligosaccharides is selected for fucosylation is different between the mammalian and bacterial enzymes. Because the two enzymes are very similar in terms of both overall structure and the formation of an α1,6-linked fucose, it is reasonable to assume that their catalytic mechanisms are also similar with the exception that the mammalian and bacterial enzymes natively act on *N*-linked oligosaccharides and chitooligosaccharides, respectively. Although it may have been thought that these two enzymes catalyze the fucosylation of the reducing terminal GlcNAc of any oligosaccharide as the acceptor, our results indicate that FUT8 does not necessarily recognize the reducing terminal of substrates.

The difference in the fucosylation of chitooligosaccharides may be attributed to how the enzymes recognize and capture the acceptors, the process of which involves the interaction of the enzyme with the substrates. Such an interaction must be conferred by the structure of the enzyme, and it seems likely that the additional domain(s) of FUT8, which is lacking in NodZ, plays a role in this distinct fucosylation. Although the exact function of the domain of FUT8, e.g. the SH3 domain, remains to be elucidated, it is possible that the domain functions to fit the substrate suitably into the active site to fucosylate the third GlcNAc of the nonreducing terminal. For example, the domain may recognize the terminal GlcNAc and then allow the third residue to be positioned in close proximity to the catalytic center. As shown by fucosylation of GN6 in Figure 5, however, other GlcNAc positions can be fucosylated to a much lesser extent in the case of a sufficiently long chitooligosaccharide, hexaose or probably longer. Thus, the reducing terminal region consisting of trisaccharide or more could also contribute slightly to selecting the position to fucosylate. As demonstrated by the present study of the fucosylation of chitooligosaccharides, the nonreducing terminal trisaccharide, but not the reducing terminal saccharide, is of critical importance for FUT8 to fucosylate the acceptor. This view is consistent with the inability of FUT8 to fucosylate chitobiose (Quesada-Vincens et al. 1997; Quinto et al. 1997; Chazalet et al. 2001). These considerations may explain how FUT8 acts on *N*-linked oligosaccharide for core fucosylation: it is probable that the enzyme determines the position of fucosylation via recognizing the β1,4-linked trisaccharide sequence, Manβ1-4GlcNAcβ1-4GlcNAcβ-, in *N*-glycans rather than the reducing terminal GlcNAc which is linked to asparagine.

It is well known that core-fucosylated *N*-glycans play various roles in the functions of glycoproteins (Miyoshi et al. 1999; Taniguchi et al. 2006). However, fucosylated chitooligosaccharides produced by FUT8 have not been reported nor have their properties been examined. The fucose residue that is added by FUT8 to relatively long chitooligosaccharides such as a pentaose and hexaose is markedly resistant to digestion by α-fucosidase whereas such resistance was not observed in the case of the shorter oligosaccharides examined (Figure 5). This inconsistent result might be due to the occurrence of a conformational difference between the molecules that protect the fucose from the glycosidase but can be formed only in the GlcNAc polymers of five residues or more. In addition, our analyses suggested that the presence of a fucose residue at the nonreducing terminal GlcNAc prevents the digestion of the fucosylated chitooligosaccharide by exo-hexosaminidase (Figure 5). It seems likely that these properties of the fucosylation by FUT8 potentially contribute to the biological stabilization of the longer chitooligosaccharides. Therefore, industrial applications of FUT8 might be useful in terms of developing new carbohydrate-based biomaterials.

The activities of FUT8 toward the chitooligosaccharide series are comparable to those of NodZ (Quesada-Vincens et al. 1997; Quinto et al. 1997; Chazalet et al. 2001), suggesting that the apparently unusual fucosylation can occur if an appropriate, native acceptor substrate is present in the organism which expresses FUT8 or its ortholog. Such a possible involvement of FUT8 in the metabolism and function of chitooligosaccharides or related types of oligosaccharides remains to be explored in addition to elucidation of its roles in the biosyntheses of *N*-glycans.

Materials and methods

Materials

GDP-β-L-fucose was purchased from Wako Pure Chemicals (Osaka, Japan). Free and pNP-labeled chitooligosaccharides

were purchased from Seikagaku Biobusiness Corporation (Tokyo, Japan). 4-Methylumbelliferyl triacetylchitotriose and 4-methylumbelliferyl *N*-acetylglucosamine were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). β-*N*-Acetylhexosaminidase (β-*N*-acetylglucosaminidase) from jack bean and α-fucosidase from bovine kidney were purchased from Sigma (St. Louis, MO). Other common chemicals were obtained from Wako Pure Chemicals, Nacalai Tesque (Kyoto, Japan) and Sigma.

Preparation of recombinant protein

The recombinant FUT8 was expressed in soluble form, with a C-terminal polyhistidine tag, using a baculovirus/insect cell expression system and purified by Ni^2 chelating affinity chromatography as described previously (Ihara et al. 2006).

Protein assay

Protein concentrations were determined using a bicinchoninic acid kit (Pierce, Rockford, IL), with bovine serum albumin as the standard.

FUT8 activity assay

α1,6-Fucosyltransferase activity was assayed using free and labeled chitooligosaccharides under modified conditions using *N*-glycans as reported previously (Uozumi, Yanagidani et al. 1996; Yanagidani et al. 1997; Ihara et al. 2006). Recombinant FUT8 was incubated at 37°C with 1 mM of acceptor substrate and 2 mM GDP-β-L-fucose as a donor in 0.1 M MES-NaOH, pH 7.0. The reactions were terminated by boiling after an appropriate reaction time, and the reaction mixtures were centrifuged at 15,000 × *g* in a microcentrifuge for 10 min. The resulting supernatants were injected to a normal phase HPLC (2695 Separation Module, Waters, Milford, MA) equipped with TSK-gel, Amide-80 (4.6 × 250 mm) (Tosoh, Tokyo, Japan), as reported about chitinase experiment previously (Abdel-Banat et al. 2002; Kabir et al. 2006). The product and substrate were separated isocratically with 70% acetonitrile as the irrigant at a flow rate of 1.0 mL/min and 30°C. UV absorbance of the column elute was monitored with a UV monitor (2487 Multi λ Absorbance Detector, Waters) for detection of free and pNP-labeled chitooligosaccharides, at 210 and 300 nm, respectively (Abdel-Banat et al. 2002; Murata et al. 2005; Kabir et al. 2006). The fluorescence of the column elute for 4MU-labeled sugars was detected with a fluorescence detector (2475 Multi λ Fluorescence Detector, Waters), at excitation and emission wavelengths of 315 nm and 380 nm, respectively (Tazawa et al. 1998).

Structural analysis of fucosylated chitooligosaccharide by exoglycosidase digestion

Fucosylated chitooligosaccharides were digested by α-fucosidase (bovine kidney, Sigma), β-*N*-acetylhexosaminidase (jack bean, Sigma) and their combination. Samples were incubated at 37°C for 24 h in 50 mM citrate buffer, pH 5.0. The reactions were terminated by boiling, and the reaction mixtures were centrifuged at 15,000 × *g* in a microcentrifuge for 10 min, after the addition of an equal amount of acetonitrile. The supernatants were then analyzed by normal phase HPLC using a TSK-gel Amide-80 column (4.6 × 250 mm) (Tosoh), and elution was performed isocratically with 76% acetonitrile at a flow rate of 1.0 mL/min and 30°C. The structures of the digested products were assigned by comparison with standard chitooligosaccharides (Seikagaku Biobusiness Corporation).

NMR experiments

NMR experiments were performed using a Bruker AVANCE-600 spectrometer (600 MHz for ^{1}H resonance frequency), and probe temperature was set at 25°C. The oligosaccharide samples were dissolved in 100% D_2O solution to be 2.5 mM (GN5F and its β-*N*-acetylhexosaminidase-digest) or 5.0 mM (GN5 and GN3). Chemical shifts were adjusted with the singlet signal of sodium 3-trimethylsilyl-1-propanesulfonate (DSS) at 0.00 ppm. To confirm the structures of GN5F and β-*N*-acetylhexosaminidase-digest of GN5F, ^{1}H-NMR, 2D ^{1}H-^{1}H COSY, 2D ^{1}H-^{13}C HSQC and 2D HSQC-TOCSY spectra were collected. For the use of NMR experiments, chitopentaose (GN5) and chitotriose (GN3) were purchased from Seikagaku Biobusiness Corporation.

MS spectrometry

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) spectra were measured using a Shimadzu AXIMA-CFR spectrometer (Kyoto, Japan) with reflectron mode, and 2,5-dihydroxybenzoic acid was employed as the matrix. Angiotensin II was used for the calibration.

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Conflict of interest statement

None declared.

Abbreviations

4MU 4-methylumbelliferone; COSY correlation spectroscopy; DSS sodium 3-trimethylsilyl-1-propanesulfonate; Fuc fucose; FUT8 α1,6-fucosyltransferase; GDP guanine nucleotide diphosphate; GN1 GlcNAc or *N*-acetylglucosamine; GN2 *N*,*N*′-diacetyl chitobiose; GN3 *N*,*N*′,*N*″-triacetyl chitotriose; GN4 *N*,*N*′,*N*″,*N*‴-tetraacetyl chitotetraose; GN5 *N*,*N*′,*N*″,*N*‴,*N*‴′-pentaacetyl chitopentaose; GN6 *N*,*N*′,*N*″,*N*‴,*N*‴′,*N*‴″-hexaacetyl chitohexaose; GNF fucosylated chitooligosaccharide; GNs chitooligosaccharides; HPLC High performance liquid chromatography; HSQC hetero-nuclear single quantum coherence; MALDI matrix-assisted laser desorption/ionization; MS mass spectrometry; NMR nuclear magnetic resonance; pNP *p*-nitrophenol; TOCSY total correlation spectroscopy; TOF time of flight; UV ultraviolet.

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