Array-assisted Characterization of a Fucosyltransferase Required for the Biosynthesis of Complex Core Modifications of Nematode N-Glycans*

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Background: The chitobiose region of nematode N-glycans can be modified with three fucose residues. Glycan arrays and other analytical techniques facilitated the definition of the biologically relevant activity of Caenorhabditis FUT-6.

Results: Glycan arrays and other analytical techniques facilitated the definition of the biologically relevant activity of Caenorhabditis FUT-6.

Conclusion: The concerted action of Caenorhabditis FUT-1, FUT-6, and FUT-8 is required for trifucosylation of worm N-glycan cores.

Significance: New approaches for studying glycans from parasitic nematodes are now possible.

Fucose is a common monosaccharide component of cell surfaces and is involved in many biological recognition events. Therefore, definition and exploitation of the specificity of the enzymes (fucosyltransferases) involved in fucosylation is a recurrent theme in modern glycosciences. Despite various studies, the specificities of many fucosyltransferases are still unknown, so new approaches are required to study these. The model nematode Caenorhabditis elegans expresses a wide range of fucosylated glycans, including N-linked oligosaccharides with unusual complex core modifications. Up to three fucose residues can be present on the standard N,N'-diacetylchitobiose unit of these N-glycans, but only the fucosyltransferases responsible for transfer of two of these (the core α1,3-fucosyltransferase FUT-1 and the core α1,6-fucosyltransferase FUT-8) were previously characterized. By use of a glycan library in both array and solution formats, we were able to reveal that FUT-6, another C. elegans α1,3-fucosyltransferase, modifies nematode glycan cores, specifically the distal N-acetylgalcosamine residue; this result is in accordance with glycomic analysis of fut-6 mutant worms. This core-modifying activity of FUT-6 in vitro and in vivo is in addition to its previously determined ability to synthesize Lewis X epitopes in vitro. A larger scale synthesis of a nematode N-glycan core in vitro using all three fucosyltransferases was performed, and the nature of the glycosidic linkages was determined by NMR. FUT-6 is probably the first eukaryotic fucosyltransferase whose specificity has been redefined with the aid of glycan microarrays and so is a paradigm for the study of other unusual glycosidic linkages in model and parasitic organisms.

Glycan arrays have begun to revolutionize the way in which we study carbohydrate-protein interactions (1) and, in combination with modern glycoanalytical and chemical glycobiological approaches (2), have transformed the experimental tools available for modern structural and functional glycobiology. However, in comparison with the examination of the binding of antibodies or lectins with glycan arrays, the determination of enzyme activities, especially of glycosyltransferases, with these platforms is not so well established, and generally only previously studied enzymes have been assessed (3–7). One exception is a recent study on the specificity of “new” glycosyltransferases from bacteria toward simple saccharide structures (8); nevertheless, the actual in vivo substrates for these enzymes remain unknown.

To date, because the focus of glycan arrays has been on mammalian glycans (9), a huge number of possible glycan structures in nature, especially non-mammalian glycans (e.g. those of model organisms or parasites) are underrepresented on existing platforms. These organisms also have or are predicted to have a variety of glycosyltransferases that have previously been unstudied or only incompletely studied; thereby, non-mammalian glycans and enzymes represent an untapped resource as well as an underestimated challenge.

One of these model organisms with a particularly rich glycomic potential is the nematode Caenorhabditis elegans (10); this species has not only become well established as a model for studies of developmental biology and innate immunity but is also related to parasitic nematodes that represent a biological burden for millions of human beings and livestock worldwide as well as for plant crops. Because nematode glycoconjugates have immunomodulatory properties (11) or are relevant in attempts to produce vaccines (12), there is a need for new approaches to study biosynthesis, binding partners, and functions of these molecules in order to identify new therapeutic targets.

Indeed, the core region of nematode N-glycans is particularly unique due to the range of so-called complex core modifications (13, 14), which represent a set of targets for lectins and potential therapeutics; not just α1,6-fucosylation of the reducing terminal (proximal) asparagine-bound N-acetylgalcosamin
Enzymatic Trifucosylation of N-Glycans

occur as in mammals, but also α1,3-fucosylation of both GlcNAc residues (proximal and distal) of the chitobiose core (Fig. 1). Thereby, nematodes express both the core α1,3-fucose on the proximal residue as also present in plants, slime molds, and other invertebrates (15) as well as a novel form of the distal N-acetylgalactosamine. Furthermore, core fucose residues can be capped with hexose; for instance, substituted and unsubstituted galactose is found linked to the proximal residue (23, 24); however, because nematode glycans are poorly represented by current glycan arrays, misleading results can be obtained regarding which glycans are the true binding partners. For instance, C. elegans was observed to bind mammalian glycans of types (fucosylated on the antennae) absent from most nematodes (20), whereas CGL2 bound mammalian glycans with (sub)terminal galactose (22); in contrast, the biological data indicated that the respective in vivo targets are core α1,3-fucosylated glycans and GalFuc epitopes actually found in nematodes (17). These unusual modifications occur not just in C. elegans but also in a number of parasitic worms, such as Ascaris suum, Hemonchus contortus, and Oesophagostomum dentatum (17, 18), and represent epitopes particular to a subset of nematodes.

These N-glycan core motifs have been found to be targets of one endogenous C. elegans galectin (LEC-6) and two nematotoxic lectins (CCL2 and CGL2) derived from fungi (19–21). However, because nematode glycans are poorly represented by current glycan arrays, misleading results can be obtained regarding which glycans are the true binding partners. For instance, CCL2 was observed to bind mammalian glycans of types (fucosylated on the antennae) absent from most nematodes (20), whereas CGL2 bound mammalian glycans with (sub)terminal galactose (22); in contrast, the biological data indicated that the respective in vivo targets are core α1,3-fucosylated glycans and GalFuc epitopes actually found in nematodes (20, 21).

The enzymology of the nematode core modifications is only partly understood. Indeed, the genome of C. elegans encodes nearly 30 potential fucosyltransferases, of which the activity of only two α1,2-, one α1,6-, and four α1,3-fucosyltransferases has been demonstrated. Initially the FUT-1 enzyme and then later three other α1,3-fucosyltransferase homologues (including FUT-6) were suggested to synthesize Lewis-type epitopes, including Le(x) and fucosylated LacdiNAc (23, 24); however, such epitopes have not, to date, been detected in C. elegans and only occur in few nematode species (25–27). Later work showed that FUT-1 is actually a core α1,3-fucosyltransferase with an unusual substrate requirement (28), whereas FUT-8, a homologue of the mammalian core α1,6-fucosyltransferase, displays a substrate specificity typical for such enzymes (29). The identity of the β1,4-galactosyltransferase encoded by the galt-1 gene, which modifies the core α1,6-fucose residue, thus forming the GalFuc epitope, was first revealed by a screen for mutants resistant to the fungal CGL2 lectin (21, 30); this indicates that non-standard techniques are essential for examining glycosylation-relevant enzymes in these organisms.

The definition of these three enzymes (FUT-1, FUT-8, and GALT-1) still left the molecular basis for the other core modifications unsolved. Neither the fucosyltransferase modifying the distal N-acetylgalactosamine nor other glycosyltransferases modifying α1,3-linked fucose or the galactosylated core α1,6-fucose have, to date, been studied. There were clues from glycomic studies of mutant worms, because not only fut-1 and fut-8 mutants are deficient in certain fucosylated N-glycans (21, 28), but also fut-6 mutant worms have an altered glycomic profile (28). Therefore, we suspected that FUT-6 may have a role in N-glycan biosynthesis independent of its ability to generate Lewis-type epitopes in vitro. Using a mixture of array- and glycomic-based approaches, we now show that FUT-6 is the enzyme that generates the distal Fucα1,3GlcNAc unit in C. elegans, a result then exploited in the chemoenzymatic synthesis of a complete trifucosylated N-glycan core. In the process, we were able to recreate the biosynthetic pathway leading in nematodes to a multiply fucosylated core recently shown to have relevance to nematotoxic lectin binding.

**EXPERIMENTAL PROCEDURES**

**Enzyme and Lectin Preparation**—HisFLAG-tagged soluble forms of C. elegans FUT-1, FUT-6, and FUT-8 were expressed in _Pichia pastoris_. The constructs were prepared directly from RT-PCR fragments in the case of FUT-1 and FUT-6, also known as CEFT1 and CEFT3 (24), or by reamplification from a previously described expression vector in the case of FUT-8 (28, 31) into a reconstructed form of pPICZαa vectors named pPICZαHisFLAG. First, the modified expression vector was obtained after two rounds of inverse PCR using KOD polymerase (Takara) to incorporate a region encoding a His tag and a FLAG tag between the region encoding the α-factor signal sequence and the Clal, PstI, and EcoRI restriction sites. Truncated open reading frames for the three fucosyltransferases (excluding the cytoplasmic and transmembrane domains) were then isolated after PCR using the following forward and reverse primers for FUT-1 (AACTGCGAGAAATCTGAACAAAGGATTGG with GCTCTAGACTAATCTAACAGGAATAGAATC), FUT-6 (AAC-TGCAGAGAGTGAAAACATAAGAGATTCG with GCTCTAG-ACAACACAAATATCTTGAAGC) and FUT-8 (TCTGGA-AAAAAGAAGACAGAAC with CGGGTACAATCTACAAAGACGTTCC). The PCR products were cut with the relevant restriction enzymes and then ligated into the pPICZ-αHisFLAG vector. Linearized constructs were integrated into the _Pichia_ genome by electroporation. All recombinant FUTs were expressed at 18 °C for 96 h prior to His tag purification using nickel affinity chromatography; purified FUTs were rebuffered and stored in 25 mM Tris-HCl, 150 mM NaCl, pH 7.0, at 4 °C. Expression of the enzymes was verified by SDS-PAGE as well as Western blotting with an anti-FLAG antibody (for

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2 The abbreviations used are: GalFuc, galactose linked β1,4 to core α1,6-fucose; LDNF, fucosylated LacdiNAc (GalNAc[β1,4(Fucα1,3)GlcNAc]; AAL, _A. aurantia_ lectin; RCA, _R. communis_ agglutinin.
FUT-6, a single band of ~45 kDa was observed upon Coomassie Blue staining; data not shown). *C. elegans* GALT-1 (30) and *Coprinopsis cinerea* lectin CCL2 (20) were the kind gifts of Dr. Markus Künzler.

**Glycan Microarray**—Microarrays were printed on Nexterion® H N-hydroxysuccinimide-activated glass slides (Schott AG, Mainz, Germany) employing a robotic non-contact spotter, sciFLEXARRAYER S11, from Scienion AG (Berlin, Germany). Droplets (2.5 nl) of each glycan solution (50 μM; glycans 1–17, Fig. 3) in sodium phosphate buffer (300 mM, pH 8.5, 0.005% Tween 20) were spatially arrayed with a spot pitch of 550 μm. Each glycan was spotted in six replicates (2 different glycans/row), producing a 1211 spot array, which was printed in 14 copies onto each slide. After printing, the slides were placed in a 75% humidity chamber (saturated NaCl solution) at 25 °C for 18 h. Unreacted N-hydroxysuccinimide groups were quenched with 50 mM ethanolamine in 50 mM sodium borate buffer, pH 9.0, for 1 h. The slides were washed with PBST (PBS solution containing 0.5% Tween 20), PBS, and water and dried in a slide spinner.

Printed synthetic glycans 1–17 were further derivatized by on-chip enzymatic modifications with recombinant glycosyltransferases. One subarray was galactosylated with a mixture containing bovine milk β1,4-galactosyltransferase (10 milliunits), alkaline phosphatase (25 milliunits), MnCl2 (5 mM), Hepes buffer (50 mM, pH 7.4), and UDP-Gal (2 mM) at 37 °C for 48 h. The introduced galactose was detected with fluorescently labeled *Ricinus communis* agglutinin RCA-555 (50 μg/ml), a lectin that recognizes β-linked galactose. Afterward, the galactosylated subarrays were incubated with a reaction mixture containing purified recombinant *C. elegans* FUT-6 (8.5 μg), MnCl2 (20 mM), MES buffer (80 mM, pH 6.5; compatible with data on the pH optimum), and GDP-Fuc (1 mM). The subarrays were then probed with fluorescently labeled *Aleuria aurantia* lectin AAL-555 (50 μg/ml), which has a broad affinity against fucose. In addition, a non-galactosylated glycan subarray was incubated with FUT-6 as above, and the introduced fucose was probed with fluorescently labeled *A. aurantia* lectin AAL-555 (50 μg/ml), which has a broad affinity against fucose. Fluorescence was measured using an Agilent G265BA microarray scanner system (Agilent Technologies, Santa Clara, CA) and quantified with ProScanArray® Express software (PerkinElmer Life Sciences), employing an adaptive circle quantitation method from 50 μm (minimum spot diameter) to 300 μm (maximum spot diameter). Average relative fluorescent unit values with local background subtraction of six spots and S.D. were reported as histograms.
the second (Hypersil ODS, Agilent). Selected purified and characterized glycans were among the substrates tested with FUT-6.

**MALDI-TOF MS**—Mass spectra were recorded using either UltrafleXtreme III or Autoflex Speed MALDI-TOF mass spectrometers, respectively, equipped with a pulsed N₂ laser (337 nm) or a SmartBEAM solid state laser and controlled by FlexControl version 3.3 software (Bruker Daltonics). MS/MS was performed by laser-induced dissociation of selected parent ions.

**In-solution Assays**—For natural pyridylaminated glycans or chemically synthesized compounds with the alkylamine linker, the reaction was carried on in a minimal mixture (2.5 μl) of glycan candidate, MnCl₂ (20 mM), MES buffer (80 mM, pH 6.5), GDP-Fuc (2 mM), and recombinant \textit{C. elegans} FUT-6, at room temperature or 37 °C overnight. Aliquots of the reaction mixture were examined by MALDI-TOF MS using 6-aza-2-thiothymine as matrix (3 mg/ml), and MS/MS (laser-induced dissociation) was performed on the products to assign the structure. As required these compounds were preincubated with FUT-1, FUT-8, and/or GALT-1 at room temperature with the requisite nucleotide sugar and Mn(II) and/or with jack bean \(\beta\)-hexosaminidase at 37 °C.

**Pyridylaminated lacto-\(\text{N}\)-neotetraose (6 pmol) was incubated with FUT-6 under similar reaction conditions prior to**

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**FIGURE 3.** Glycan array screening for FUT-6 substrates. \(A\), \(\text{N}\)-glycan microarray after enzymatic reaction with bovine milk \(\beta\)-1,4-galactosyltransferase followed by \textit{C. elegans} \(\alpha\)-1,3-fucosyltransferase (FUT-6) to generate Lewis x type epitopes (boxed inset) on the antennae of complex and hybrid \(\text{N}\)-glycans. \(a\), non-modified \(\text{N}\)-glycan structures originally printed on the microarray. \(b\), \(\text{N}\)-glycan microarray images after galactosylation incubated with the galactose-recognizing lectin from \textit{R. communis} (RCA-555). \(c\), images of the galactosylated \(\text{N}\)-glycan microarray after FUT-6 reaction and incubation with the fucose-recognizing lectin from \textit{A. aurantia} (AAL-555). \(d\), fluorescence intensities of the galactosylated array after incubation with RCA-555. \(e\), fluorescence intensities with AAL-555 after GalT and FUT-6 incubation. \(B\), \(\text{N}\)-glycan microarray after enzymatic reaction with CeFUT-6 without preincubation with galactosyltransferase. \(a\), \(\text{N}\)-glycan microarray images after incubation with fucose-recognizing lectins (AAL and CCL2) and anti-HRP-555 antibody. \(b\), fluorescence intensities after incubation with AAL-555. \(c\), fluorescence intensities after incubation with CCL2–647. Compounds 19 and 21, already containing fucose, were included as controls; however, only the latter (LDNF) reacted with CCL2. Each histogram represents the average relative fluorescent unit values for six replicates with the S.D. Red triangles, fucose; yellow circles, galactose; blue squares, \(\text{N}\)-acetylglucosamine; green circles, mannose.
isocratic reversed phase HPLC (32). For Lewis-type fucosylation, dabsylated Gly-Glu-Asn-Arg-glycopeptides derived from bovine fibrin (0.25 nmol), either the asialo Gaal Gal glycopeptide or the asialoagalacto glycopeptide incubated with bovine galactosyltransferase in the presence of UDP-GalNac (to form dabsyl-βGnβGn with terminal GalNac residues), were also incubated under the same conditions prior to product analysis by MALDI-TOF MS using α-cyanohydroxycimetic acid as matrix. For assessment of core fucosylation activity, 5 nmol of the dabsyl asialoagalacto glycopeptides were remodeled with FUT-8 and then hexosaminidase to yield dabsyl-MMF6 (Man1GlcNAc2Fuc1), prior to α-mannosidase digestion to yield dabsyl-00F6 (Man1GlcNAc2Fuc1) and incubation with FUT-6 (see also Fig. 5).

For fucosylation of trisaccharide 1 (see Scheme 1) on a large scale for NMR, 500 µl of a solution of trisaccharide 1 (1.0 mg, 1.5 µmol), GDP-Fuc (1.2 mg, 1.9 µmol), C. elegans FUT-6 (50 µg), and MnCl2 (20 mm) in MES buffer (80 mm, pH 6.5) were incubated at room temperature for 72 h. The resulting mixture was heated at 95 °C for 5 min and centrifuged, and the solution was purified on graphitized carbon (SulpelClean™ ENVITM Carb cartridges, Sigma-Aldrich). The fucosylated tetrasaccharide 23 was freeze-dried to obtain the title compound as a white powder (1.1 mg, 1.34 µmol, 89%).

HPLC Analysis of Pyridylaminated Products—The examination of FUT-6 modified pyridylaminated lacto-β-neotetraose was carried out on the reversed phase HPLC. A Hypersil ODS column (250 × 4.0 mm; Agilent) was used with 0.1 m ammonium acetate, pH 4.0 (buffer A) and 30% methanol (buffer B). The gradient of buffer B was applied as follows: 0–11 min, 5% B; 11–12, 5–50% B; 12–15 min, 50% B; 15–16 min, 50–0% B; 16–21 min, 0% B.

NMR—Compounds were freeze-dried and dissolved in deuterium oxide (D2O) for recording 1H NMR spectra. Nuclear magnetic resonance experiments were acquired on a Bruker 500-MHz spectrometer, and chemical shifts (δ) are given in ppm relative to the residual signal of the solvent used (D2O 4.79 ppm).

RESULTS

Array-based Screening of Fucosyltransferase Specificities—As initial glycomic evidence from single mutants (28) suggested that the C. elegans α1,3-fucosyltransferase FUT-6 might have an activity other than its known ability to synthesize Leα as well as fucosylated LacdiNac (LDNF) in vitro (24), a finding that was reproduced by our own assays (Fig. 2), we considered new approaches to reveal the in vivo specificity of this enzyme. We have previously tested a new N-glycan array with two fucosyltransferases with known specificity, the core α1,3-fucosyltransferase FucTA from Arabidopsis thaliana, and the core α1,6-fucosyltransferase FUT-8 from C. elegans (7). On-array fucosylation by both these enzymes was easily assessed using the fucose-specific lectin from A. aurantia (AAL). Furthermore, human asialotransferrin fucosylated on the LacNac antennae in vitro by FUT-6 was previously shown to bind AAL (33). Therefore, we investigated the impact of His tag-purified recombinant FUT-6 on AAL binding to the array with and without prior incubation with β1,4-galactosyltransferase (Fig. 3) because pregalactosylation is a requirement for synthesis of Leα; thereby, FUT-6 was incubated with galactosylated and non-galactosylated forms of the N-glycan array, which contained paucimannosidic glycans cores lacking non-reducing GlcNac as well as mono-, bi-, tri-, and tetra-antennary N-glycans, the latter representing also galactosyltransferase substrates. The efficiency of pregalactosylation was assessed using the galactose-specific lectin from R. communis (RCA). As expected from the previous in vitro data on FUT-6, galactosylated structures 7–17 (whose galactosylation status was proven by RCA binding) gained AAL reactivity upon incubation with FUT-6 (Fig. 3A). Furthermore, non-galactosylated paucimannosidic structures (compounds 1, 2, and 5 on the arrays either with or without preincubation with galactosyltransferase; Fig. 3, A and B) lacking the α1,6-arm were surprisingly also AAL-positive. On the non-galactosylated array, additional FUT-6 substrates were those with non-reducing GlcNac on the α1,3-arm but lacking the α1,6-mannose linked to the core β1,4-mannose (8 and 10; Fig. 3B). The spot corresponding to galactosylated N-glycan 18, included as a positive substrate control on an otherwise non-galactosylated array, was recognized by AAL after incubation with FUT-6 due to the expected formation of antennal Leα. The multiantennary non-galactosylated glycans and the hybrid-like structures with an α1,6-mannose were not modified by FUT-6. None of the products of FUT-6 were bound by anti-HRP or by the fungal CCL2 lectin, both of which can recognize core α1,3-fucose; only the preformed LDNF trisaccharide 21 was recognized by CCL2. Therefore, we concluded that FUT-6 not only generates Leα epitopes on the antennae of glycan substrates in vitro but transfers fucose to another position on selected N-glycans. However, it cannot form the anti-HRP epitope, which is dependent on α1,3-fucosylation of the proximal core GlcNac.

Glycomic Analysis of Fucosyltransferase Mutants—Additional clues regarding the specificity of FUT-6 were expected by
FIGURE 5. In-solution modification of pyridylaminated natural glycans and a remodeled dabsylated glycopeptide with *C. elegans* FUT-6. A–G, selected two-dimensional HPLC fractions of N-glycans from double mutant strains of *C. elegans* were analyzed by MALDI-TOF MS before and after incubation with recombinant FUT-6; the analyzed glycans were detected as [M + H]⁺, and transfer of fucose to four of the glycans (B, C, D, and G) is indicated by a Δm/z of 146. H–M, MALDI-TOF MS analysis of a dabsylated asialoglycopeptide derived from bovine fibrin (GalGal) after treatment with glycosidases and with recombinant *C. elegans* core α1,6-fucosyltransferase FUT-8 to yield OOF⁶ (H–L) prior to incubation with recombinant *C. elegans* FUT-6 (M); whereas GalGal itself is a substrate for the Lewis-type fucosylation by FUT-6 (Fig. 2), the remodeling to OOF⁶ is necessary to detect the core fucosylation activity because GnGn and MM glycopeptides are not substrates for this enzyme (data not shown). Red triangles, fucose; yellow circles, galactose; blue squares, N-acetylglucosamine; green circles, mannose.
analyzing C. elegans mutants lacking this enzyme. The \textit{fut-6} single mutant was previously shown to lack tetrafucosylated glycans in the peptide-N-glycanase A-released pool but still possessed trifucosylated glycans (28). Therefore, various double mutants lacking two fucosyltransferases each were also prepared: \textit{fut-1}/\textit{fut-6} (previously shown to be completely resistant to CCL2 (20)), \textit{fut-6}/\textit{fut-8}, and \textit{fut-1}/\textit{fut-8}. Glycomic analyses indicated that the latter two mutants had maximally two fucose residues on their N-glycans (Fig. 4), whereas the \textit{fut-1}/\textit{fut-6} still had traces of trifucosylated N-glycans. This is a further suggestion of an N-glycan-modifying activity of FUT-6.

In-solution Assays with Various Substrates—Although the \textit{in vitro} lectin-based assay was a first indication of the unique specificity of FUT-6 and the glycomic analyses were in accordance with a role of this enzyme in the modification of N-glycans \textit{in vivo}, further verification was required in order to localize the new glycosidic linkage formed by this enzyme. Therefore, a range of substrates suitable for “in-solution” studies were examined: specifically, pyridylaminated forms of glycans isolated from C. elegans, remodeled dabsylated glycopeptides, and chemically synthesized glycans of the form (i.e. functionalized with an alkylamine spacer) also used for printing the N-glycan array.

A working hypothesis was that FUT-6 transfers fucose to the distal GlcNAc of N-glycans; this type of fucose modification was presumed missing from \textit{fut-1}/\textit{fut-6} mutants but was previously shown to be overrepresented in a hex-2/\textit{hex-3} strain (17). Therefore, N-glycans from these mutants presumed to be biosynthetic intermediates were incubated with purified FUT-6. In particular, two isomers of core α1,6-fucosylated glycans with the compositions Hex$_1$HexNAc$_2$Fuc$_1$ and Hex$_2$HexNAc$_2$Fuc$_1$ as well as two structures of the form Hex$_2$–3HexNAc$_2$Fuc$_1$ (one of which is an α1,3-mannosidase digestion product of a natural glycan) were tested (Fig. 5, A–G). Only four of these seven glycans were actual acceptor substrates for the \textit{in vitro} enzymatic reaction; the common element in these substrates was an absence of an α1,6-mannose, but the presence of the β1,4-mannose, on the core region. Unfortunately, MS/MS of these difucosylated products did not result in the appearance of a key fragment for the transferred fucose (data not shown). Notably, a glycan treated with α1,3-mannosidase but retaining the α1,6-mannose is not accepted by FUT-6 (Fig. 5E).

Based on these data, a dabsylated glycopeptide derived from bovine fibrin was remodeled by degalactosylation and core α1,6-fucosylation followed by removal of the antennal GlcNAc residues and of the α1,3/6-mannose residues (Fig. 5, H–L). The resultant glycopeptide carrying a Man$_1$GlcNAc$_2$Fuc$_1$ glycan was a substrate for recombinant FUT-6 (Fig. 5I); MS/MS showed a low intensity fragment of \( m/z \) 512, indicative that the transferred fucose is associated with the Man$_1$GlcNAc$_2$ region but not with the reducing terminal GlcNAc (data not shown).

For exact analysis of the position of the transferred fucose, a selection of glycans with an alkylamine linker on the reducing end (used also for printing the glycan arrays) was employed for reactions in solution. In total, four substrates (compounds 1, 2, 8, and 10), chosen on the basis of a positive result with the glycan array (Fig. 3), were tested, and all were found to be fuco-
sylated by FUT-6 in vitro as shown by mass spectral data (Fig. 6). MS/MS of the simplest structure resulted in a set of fragments that were more informative than those of the pyridylaminated and dabsylated products; thereby, the localization of the transferred fucose to the distal GlcNAc rather than to the β-linked mannose was more certain. In particular, the simultaneous appearance of Hex1HexNAc1Fuc1 (m/z 511 as [M + H]+ and m/z 533 as [M + Na]+) and alkylaminated HexNAc2Fuc1 (m/z 655 as [M + H]+ and m/z 677 as [M + Na]+) ions as fragments of the fucosylated “monoa antennary” compound 1 was the most promising piece of evidence that the fucosylation by FUT-6 took place on the distal GlcNAc (Fig. 6, f and l).

Characterization of an Enzymatic Product by NMR—In order to more definitively verify the linkage of the transferred fucose, the chemically synthesized trisaccharide 1 (Man3GlcNAc2) was fully fucosylated by FUT-6 (Scheme 1) and the fucosylated product 23 analyzed by 1H NMR; the chemical shifts for each proton of both compounds are listed in Table 1. The introduction of one fucose moiety was confirmed by the appearance of a new anomeric proton at δ 5.16 ppm with a coupling constant of \( J_{H-1,H-2} \) = 3.9 Hz, characteristic of an \( \alpha \)-glycosidic bond. The characteristic signal of H-6 protons from the newly introduced fucose appeared as a doublet at δ 1.08 ppm with a coupling constant of 6.6 Hz and an integration for three protons. The complete assignment of the 1H NMR spectra of the fucosylated product and the trisaccharide 1 was achieved by one- and two-dimensional NMR experiments, using 1H–1H COSY, 1H–13C heteronuclear single quantum correlation spectroscopy, total correlation spectroscopy, and NOESY. A closer look at the protons of each sugar (Scheme 1, A–G) showed that the H-3 signal of the distal GlcNAc was shifted from 4.69 ppm (GlcNAc A) in trisaccharide 1 to δ 3.96 ppm (GlcNAc E) in the fucosylated product 23, indicating fucosylation at this position. Furthermore, an NOE effect was observed between the H-1 of the fucose at 5.16 ppm and the H-3 of the GlcNAc E at 3.96 ppm, confirming the \( \alpha \)1,3 linkage.

Biosynthetic Pathways for Complex Core Modifications—To investigate the basis for the multifucosylated core, the chemically synthesized compound 10 was first \( \alpha \)1,6-fucosylated by C. elegans FUT-8 and sequentially remodeled using various recombinant glycosyltransferases from C. elegans as well as jack bean hexosaminidase. In conclusion, two biosynthesis pathways were revealed, leading to the final formation of a trifucosylated N-glycan core: (a) hexosaminidase → FUT-1 → FUT-6 and (b) FUT-6 → hexosaminidase → FUT-1 (Fig. 7, A and B). FUT-1 only worked on hexosaminidase-processed substrates lacking non-reducing terminal GlcNAc, whereas FUT-6 did not have this restriction. The results also showed that there was no specific order of \( \alpha \)1,3-fucosylation on the two core GlcNAcs.

Considering that many core fucose residues are also capped with galactose, it was also of interest to examine the point at which the core \( \alpha \)1,6-fucose can be galactosylated, using the only proven nematode galactosyltransferase, C. elegans GALT-1 (30). Galactose was only transferred by GALT-1 to the core \( \alpha \)1,6-fucosylated (Fig. 7, C and D); no further galactosylation appeared to occur on any of the \( \alpha \)1,3-linked fucose residues. Furthermore, the action of GALT-1 was prevented by preincubation with either FUT-1 or FUT-6. The downstream \( \alpha \)1,3-fucosylation by FUT-1 and FUT-6 on substrates carrying the GalFuc epitope followed pathways similar to those of the non-galactosylated glycans as described above.

MS/MS of glycan products (Fig. 8) was then employed to examine the location of the transferred fucose residues. The core \( \alpha \)1,6-fucose introduced by FUT-8 is always associated with the reducing terminal GlcNAc and the alkylamine linker; this results in the HexNAc2Fuc1-(CH2)5NH2 ion (m/z 475; Fig. 8, B–F) unless it is further modified with GALT-1 (G–K). Difucosylated compounds resulted from the action of FUT-8 and FUT-1 display diagnostic ions such as HexNAc3Fuc2-(CH2)5NH2 (m/z 621; Fig. 8, E and F) and, when galactosylated by GALT-1, Hex5HexNAc2Fuc3-(CH2)5NH2 (m/z 783; F and K). FUT-6-modified compounds possess either Hex3HexNAc2Fuc1 ion (m/z 696; Fig. 8, D, F, I, and K) or Hex5HexNAc2Fuc1 ion (m/z 899; C and H). The trifucosylated final products display the Hex5HexNAc4Fuc3-(CH2)5NH2 fragment (m/z 970; Fig. 8F) or its galactosylated form Hex5HexNAc4Fuc3-(CH2)5NH2 (m/z 1132; K).

Formation of a Trifucosylated N-Glycan Core in Vitro—One of the biosynthetic pathways toward the formation of the trifucosylated N-glycan core (FUT-8 → FUT-6 → hexosaminidase → FUT-1; Fig. 9) was performed on a larger scale, starting with compound 10 (0.9 mg, 0.87 μmol). The sequential introduction of the fucose residues was easily monitored by following the NMR chemical shifts of the H-1 and H-6 protons of the fucose residues with different linkages (Fig. 9 and Table 2). Initially, the pentasaccharide 10 was treated with FUT-8 and GDP-Fuc to introduce the core \( \alpha \)1,6-fucose, the fucosylated product 24 was purified, and 1H NMR was recorded. The H-1 of the fucose appeared as a doublet at δ 4.90 ppm with a coupling constant of \( J_{H-1,H-2} = 3.75 \) Hz. The characteristic doublet corresponding to H-6 of the newly introduced fucose appeared at δ 1.22 ppm.

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**TABLE 1**

| δ ppm | GlcNAc (A) | GlcNAc (B) | Man (C) | GlcNAc (D) | GlcNAc (E) | Man (F) | Fucose (G) |
|-------|------------|------------|---------|------------|------------|---------|------------|
| H-1   | 4.42       | 4.53       | 4.69    | 4.42       | 4.54       | 4.68    | 5.16       |
| H-2   | 3.63       | 3.71       | 3.99    | 3.64       | 3.91       | 3.98    | 3.67       |
| H-3   | 3.62       | 3.69       | 3.58    | 3.62       | 3.96       | 3.58    | 3.94       |
| H-4   | 3.54       | 3.69       | 3.5    | 3.53       | 3.88       | 3.45    | 3.72       |
| H-5   | 3.43       | 3.53       | 3.35    | 3.43       | 3.59       | 3.29    | 4.49       |
| H-6a,b| 3.59, 3.77 | 3.70, 3.82 | 3.66, 3.86 | 3.58, 3.78 | 3.70, 3.85 | 3.59, 3.87 | 1.08 |
| CH₃   | 1.08       |           |         |            |            |         |            |
This compound was subjected to reaction with FUT-6 to yield compound 25, and the introduction of an α1,3-fucose in the distal GlcNAc could be demonstrated by the appearance of a doublet at δ 5.19 ppm with a coupling constant of $J_{H-1,H-2} = 3.7$ Hz, corresponding to H-1. Additionally, a doublet corresponding to H-6 appeared at δ 1.15 ppm. This difucosylated glycan 25 was treated with jack bean hexosaminidase. The removal of the terminal GlcNAc is demonstrated by the disappearance of a doublet at δ 4.54 ppm corresponding to H-1 and one singlet at δ 2.05 ppm corresponding to the acetyl group of this residue (glycan 26). Finally, the reaction with FUT-1 led to the formation of the trifucosylated core structure 27. The introduction of core α1,3-fucose was detected in the 1H NMR spectra by the appearance of a doublet at δ 5.21 ppm with a coupling constant of $J_{H-1,H-2} = 4.0$ Hz corresponding to H-1 and a doublet at δ 1.26 ppm for the H-6 of the fucose. The chemical shifts for this ...
trifucosylated structure are in agreement with those reported previously for a similar compound prepared by chemical synthesis (34).

**DISCUSSION**

**Fucosyltransferase Substrate Screening**—The substrate specificities of glycosyltransferases can be very subtle, and apparently small changes to glycan structures distant to the site of glycosylation can have an impact on whether a glycan is an acceptor or not; whether or not a protein-linked glycan is modified depends also on factors such as accessibility on the protein surface, glycosyltransferase expression levels, and the concentrations of the nucleotide sugar donors. The traditional view (35) was that for each glycosidic linkage, there is a specific enzyme (“one linkage-one enzyme”). However, it later became obvious that, in many circumstances, multiple enzymes can form the same linkage, or one enzyme may be able to form multiple related linkages. This scenario is shown by the activities of the six proven human fucosyltransferases forming Lewis epitopes, one of which (Fuc-TIII) can form either 1,3 or 1,4 linkages, dependent on the substrate (36). In the past, screening of glycosyltransferase specificities was often unsystematic and reliant on the availability of natural sources of glycosylation precursors; generally, even for invertebrate enzymes, such as FUT-6, previously used substrates were those based on acceptors for mammalian enzymes, which led to misleading results.

However, the development of glycan arrays opens up new possibilities for examining these enzymes but to date has been (in terms of eukaryotic systems) limited to studying rather well characterized examples, such as mammalian fucosyl- and sialyltransferases or enzymes involved in plant cell wall biosynthesis (37–40). On the other hand, glycosyltransferases have been useful in synthesis of glycan libraries subsequently printed onto arrays (41).

Recently, some of us have developed a systematic array of N-glycans and N-glycan-like molecules on the basis of printing alkylamine-modified chemically synthesized oligosaccharides onto glass surfaces. These have been successfully appraised also using glycosyltransferases (a galactosyltransferase, a sialyltransferase, and two core fucosyltransferases) of known specificities, employing lectins and antibodies as detection reagents (6, 7, 42). A particular challenge, therefore, was to examine a glycosyltransferase from a model organism with an in vitro substrate specificity apparently not matching its role in vivo.

As summarized above, the α1,3-fucosyltransferase homologue FUT-6 from *C. elegans* can act as a Lewis-type enzyme *in vitro*, but a deletion in the *fut-6* gene results in a loss of tetrafucosylated non-Lewis-type N-glycans *in vivo*; among the double fucosyltransferase mutants, no more than two fucose residues are present in the *fut-6; fut-8* mutant. Although suggestive of a role for FUT-6 in N-glycan processing *in vivo*, our data resulting from on-chip fucosylation are the first to show its unique

![Image](https://example.com/figure8.png)

**FIGURE 8.** MS/MS of substrates and products elucidating the formation of trifucosylated glycans. Spectra A–F display the fragmentation patterns of the non-galactosylated glycans HexHexNAc2Fuc0–3-(CH2)5NH2 with m/z 1059, 1205, 1351, 1148, and 1294 (see Fig. 7, A and B), whereas spectra G–K display those of the galactosylated glycans Hex3HexNAc2Fuc0–3-(CH2)5NH2 with m/z 1367, 1513, 1310, and 1456 (see Fig. 7, C and D). All the ions annotated are [M–Na]+, and predicted structures of the key ions are shown in Consortium for Functional Glycomics format; the alkylamine linker, -(CH2)5NH2, is represented by a short vertical bar at the right of N-acetylglucosamine. 475, HexNAc,Fuc,CH3,NH2; 621, HexNAc,Fuc,CH3,NH2; 637, HexNAc,Fuc,CH3,NH2; 678, HexNAc,Fuc,CH3,NH2; 696, HexNAc,Fuc,CH3,NH2; 763, HexNAc,Fuc,CH3,NH2; 824, HexNAc,Fuc,CH3,NH2; 899, HexNAc,Fuc,CH3,NH2; 970, HexNAc,Fuc,CH3,NH2; 1132, HexNAc,Fuc,CH3,NH2; 1324, HexNAc,Fuc,CH3,NH2. Red triangles, fucose; yellow circles, galactose; blue squares, N-acetylglucosamine; green circles, mannose.
Preparation of the trifucosylated compound 27. Shown is the synthetic pathway toward the formation of the trifucosylated core N-glycan 27 (left) and a comparison of the significant regions of the $^1$H NMR spectra of the fucosylated N-glycans. Chemical shifts corresponding to annotated residues A, B, C, and D are highlighted in the relevant parts of the spectra. The NHAc chemical shifts are those of the three (compounds 24 and 25) or two (26 and 27) GlcNAc residues, whereas those in the H-6 fucose region derive from the one (24), two (25 and 26), or three (27) fucose residues in each compound. Red triangles, fucose; yellow circles, galactose; blue squares, N-acetylglucosamine; green circles, mannose.

| Table 2 | Selected $^1$H NMR data for di- and trifucosylated compounds 24–27 (see also Fig. 9) |
|---------|-----------------------------------------------------------------------------------|
| Compound | Anomeric region                                                                 | NHAc                  | H-6 Fuc               |
| 24       | 5.11 (s, 1H, H-1$_{1–3}$Man), 4.90 (d, J = 3.7 Hz, 1H, H-1$_{1–4}$Man), 4.69 (s, 1H, H-1$_{1–6}$Fuc), 4.65 (d, J = 7.8 Hz, 1H, H-1$_{1–4}$Man), 4.54 (d, J = 8.3 Hz, 1H, H-1$_{1–6}$GlcNAc), 4.48 (d, J = 8.2 Hz, 1H, H-1$_{1–6}$GlcNAc) | 2.07 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H) | 1.22 (d, J = 6.6 Hz, 3H) |
| 25       | 5.19 (d, J = 3.7 Hz, 1H, H-1$_{1–3}$Man), 5.08 (s, 1H, H-1$_{1–4}$Man), 4.91 (d, J = 3.7 Hz, 1H, H-1$_{1–4}$Man), 4.70 (s, 1H, H-1$_{1–4}$Man), 4.67–4.62 (m, 2H, H-5Fuc, H-1$_{1–2}$GlcNAc), 4.54 (d, J = 8.5 Hz, 1H, H-1$_{1–6}$GlcNAc), 4.48 (d, J = 8.2 Hz, 1H, H-1$_{1–6}$GlcNAc) | 2.06 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H) | 1.22 (d, J = 6.6 Hz, 3H), 1.15 (d, J = 6.4 Hz, 3H) |
| 26       | 5.20 (d, J = 3.6 Hz, 1H, H-1$_{1–3}$Man), 5.07 (s, 1H, H-1$_{1–4}$Man), 4.92 (d, J = 3.8 Hz, 1H, H-1$_{1–4}$Man), 4.70 (s, 1H, H-1$_{1–4}$Man), 4.68–4.63 (m, 2H, H-5Fuc, H-1$_{1–2}$GlcNAc), 4.49 (d, J = 8.0 Hz, 1H, H-1$_{1–6}$GlcNAc), 4.48 (d, J = 8.2 Hz, 1H, H-1$_{1–6}$GlcNAc) | 2.06 (s, 3H), 2.03 (s, 3H) | 1.22 (d, J = 6.5 Hz, 3H), 1.15 (d, J = 6.5 Hz, 3H) |
| 27       | 5.21 (d, J = 4.0 Hz, 1H, H-1$_{1–3}$Man), 5.14 (d, J = 3.5 Hz, 1H, H-1$_{1–3}$Man), 5.06 (s, 1H, H-1$_{1–3}$Man), 4.95 (d, J = 3.6 Hz, 1H, H-1$_{1–4}$Man), 4.69 (s, 1H, H-1$_{1–4}$Man), 4.68–4.62 (m, 3H, H-2H$_{5}$Fuc, H-1$_{1–2}$GlcNAc), 4.47 (d, J = 7.9 Hz, 1H, H-1$_{1–6}$GlcNAc) | 2.03 (s, 3H), 2.01 (s, 3H) | 1.26 (d, J = 6.5 Hz, 3H), 1.22 (d, J = 6.6 Hz, 3H), 1.14 (d, J = 6.5 Hz, 3H) |
capacity to transfer fucose to the distal GlcNAc of N-glycans in vitro.

These data were then confirmed in assays with a variety of substrates in solution, followed by product characterization by MALDI-TOF MS/MS and NMR. The common element in both the N-glycan and Lewis-type acceptors for FUT-6 appears to be a Hex\(^1\)/H\(^9\)252\(^1\),4GlcNAc unit, where the hexose can be either mannose or galactose. Interestingly, FUT-6 can transfer two fucoses to a galactosylated monoantennary N-glycan, one to the distal GlcNAc and one to the antenna (data not shown). However, only the transfer to the distal GlcNAc is meaningful in terms of the glycome of C. elegans, and, of those structures tested, only monoantennary N-glycans, lacking non-reducing terminal galactose as well as the \(\alpha_1,6\)-mannose of the trimannosyl core, are biologically significant substrates for FUT-6. The unusual specificity of this enzyme for such N-glycans correlates with the structures of the distally fucosylated monoantennary N-glycans observed in the hex-2/hex-3 mutant (17); our data also suggest a role for a Golgi-localized mannosidase activity removing the core \(\alpha_1,6\)-mannose during the biosynthesis of FUT-6-modified C. elegans N-glycans.

N-Glycan Core Modifications in Nematodes—With the knowledge that FUT-6 can fucosylate the distal GlcNAc of the N-glycan core, it is easier to understand the enzymatic biosynthesis pathways of highly fucosylated N-glycan cores in C. elegans (13, 43). Thereby, the two fucosyltransferases (FUT-1 and FUT-8) are defined to solely fucosylate the proximal GlcNAc, whereas FUT-6 is the third core-modifying fucosyltransferase; thus, the order of fucosylation was of interest. Because the other \(\alpha_1,3\)-fucosyltransferase, FUT-1, can act on products of the core \(\alpha_1,6\)-fucosyltransferase FUT-8, but FUT-8 cannot act on FUT-1 products (31), the same rule might apply to FUT-6. Therefore, the order of fucosylation was tested on FUT-6 and FUT-8. Indeed, FUT-6-processed glycans (products of 1, 2, 8, and 10) cannot be modified by FUT-8 (data not shown), but FUT-8 products are acceptors for FUT-6. On the other hand, the action of GALT-1, which synthesizes the ligand for two galectins (the endogenous nematode LEC-6 and the fungal nematoxic CGL2), is inhibited when either distal or proximal \(\alpha_1,3\)-fucose is present. These reactions are summarized in Fig. 10; the substrate status of tested compounds is also shown in Table 3.

However, fucosylation of the core is not the end of the story. FUT-6-processed substrates are then available for the action of a putative \(\alpha_1,2\)-galactosyltransferase-generating \(\alpha\)-galactosylated fucose (Gal\(\beta\)1,2Fuc\(\alpha\)1,3) epitope (17), which differs from the proximal GlcNAc-linked GalFuc (Gal\(\beta\)1,4Fuc\(\alpha\)1,6) epitope.
found in *C. elegans* as well as in planaria or mollusks. It is possible that an appropriate mix of approaches (analysis of mutants and use of substrates first identified by array screening) will be necessary for the identification of further glycosyltransferases required for the modification of glycans in *C. elegans* and other “lower” model or parasitic organisms.

Indeed, the postulated core modification pathways are not just applicable to the model organism *C. elegans* but also take place in parasitic nematodes, such as *A. suum*, *H. contortus*, and *O. dentatum* (17, 18). Therefore, based on glycomic data, we presume that the distal fucosylation reaction performed by FUT-6 is specific to a subset of nematodes. However, trifucosylation has not been detected to date in a number of other nematode species, including *Trichinella spiralis* (44) and *Onchocerca volvulus* (45); this appears to correlate with the phylogeny of nematode glycosyltransferases because obvious FUT-6 orthologues are lacking in the trichinellid and filarial species (data not shown).

**Perspectives**—Parasites have a high impact on quality of life as well as on agricultural productivity; on the other hand, because the immune systems evolved while being subject to the selection pressure of helmint infections, the absence of parasites is possibly associated with the huge increase in allergies and autoimmunity (46). Indeed, whereas glycosylation may play a role in the efficacy of vaccination against nematodes in farm animals (47), nematode glycans have been implicated in the effects of these organisms on mammalian immune systems (48) and may be relevant to the effects of controversial therapies against autoimmune diseases, in which patients ingest nematode eggs (49). It is of interest that trifucosylated glycans, similar to those prepared during the present study in vitro, are present on the H11 glycoprotein of *H. contortus*, a known vaccine antigen candidate (18). Thus, the definition of parasite glycan modification pathways and the utilization of the relevant enzymes may not only aid the identification of vaccine targets or the preparation of recombinant vaccine antigens with a more natural glycosylation pattern but also facilitate the production of immunomodulatory factors; furthermore, synthesis of trifucosylated glycan structures required for definition of the natural specificity of endogenous carbohydrate-binding proteins in nematodes or of nematotoxic lectins becomes feasible. Indeed, because a *fut-1/fut-6* double mutant is completely resistant to nematoxic CCL2, whereas *fut-1* and *fut-6* single mutants are either only partially or not resistant (20, 21), the FUT-6-mediated modification of nematode N-glycans is (either on its own or in combination with other residues) an interesting target for anthelminthic agents.

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