Purification, cDNA Cloning, and Expression of GDP-L-Fuc:Asn-linked GlcNAc α1,3-Fucosyltransferase from Mung Beans

(Received for publication, March 3, 1999, and in revised form, April 28, 1999)

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Substitution of the asparagine-linked GlcNAc by α1,3-linked fucose is a widespread feature of plant as well as of insect glycoproteins, which renders the N-glycan immunogenic. We have purified from mung bean seedlings the GDP-L-Fuc:Asn-linked GlcNAc α1,3-fucosyltransferase (core α1,3-fucosyltransferase) that is responsible for the synthesis of this linkage. The major isoform had an apparent mass of 54 kDa and isoelectric points ranging from 6.8 to 8.2. From that protein, four tryptic peptides were isolated and sequenced. Based on an approach involving reverse transcriptase-polymerase chain reaction with degenerate primers and rapid amplification of cDNA ends, core α1,3-fucosyltransferase cDNA was cloned from mung bean mRNA. The 2200-base pair cDNA contained an open reading frame of 1530 base pairs that encoded a 510-amino acid protein with a predicted molecular mass of 56.8 kDa. Analysis of cDNA derived from genomic DNA revealed the presence of three introns within the open reading frame. Remarkably, from the four exons, only exon II exhibited significant homology to animal and bacterial α1,3/4-fucosyltransferases which, though, are responsible for the biosynthesis of Lewis determinants. The recombinant fucosyltransferase was expressed in SF21 insect cells using a baculovirus vector. The enzyme acted on glycopeptides having the glycan structures GlcNAcβ1-2Manα1-3GlcNAcβ1-2Manα1-6Manβ1-4GlcNAcβ1-3Manα1-6Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ1-Asn, and GlcNAcβ1-2Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ1-Asn but not on, e.g., N-acetyllactosamine. The structure of the core α1,3-fucosylated product was verified by high performance liquid chromatography of the pyridylaminated glycan and by its insensitivity to N-glycosidase F as revealed by matrix-assisted laser desorption/ionization time of flight mass spectrometry.

The most characteristic features of asparagine-linked oligosaccharides from plants are the substitution of the core pentasaccharide by xylose and α1,3-linked fucose (1, 2). The resulting heptasaccharide “MMXF3” (Fig. 1) very often constitutes the main oligosaccharide species on a plant glycoprotein (3, 4). According to their biosynthesis, these structures are classified as complex-type N-glycans, even though the terms paucimannosidic or truncated N-glycans appear to be more justified. The α-mannosyl residues may, however, be substituted by GlcNAc and these GlcNAc residues may be further decorated by galactose and fucose to form the same structure as the human Lewis a epitope (Fig. 1) (5, 6).

The antigenicity of “paucimannosidic” plant N-glycans is well documented (7–11). Since both xylose and core α1,3-fucose are not seen in mammalian glycoproteins they may form the key component of epitopes for carbohydrate-reactive antibodies (9, 10, 12). There is, however, evidence that the α1,3-linked fucosyl residue is the predominant antibody binding structural element (3, 8, 11, 13). Due to the ubiquitous occurrence of such paucimannosidic N-glycans throughout the plant kingdom, they are responsible for the frequently observed cross-reactivity of antibodies raised against plant glycoproteins and are therefore termed “cross-reactive carbohydrate determinants” (12, 14, 15). Anti-cross-reactive carbohydrate determinants antibodies of the IgE class have been found in sera of many allergic patients (8, 11, 13, 14, 16, 17). While the clinical role of cross-reactive carbohydrate determinants remains controversial, they are suspected to obscure (at least in vitro) allergy diagnosis. Anti-cross-reactive carbohydrate determinants antibodies will also react with many insect glycoproteins such as honeybee venom phospholipase A2 or neuronal membrane glycoproteins from insect embryos because insects, like plants, are capable of synthesizing the core α1,3-fucose epitope (3, 11–13, 18, 19).

In contrast to the blood group-related fucosyltransferases which act on the nonreducing terminus of N-glycans, O-glycans, or glycolipids (20), core fucosyltransferases have received little attention. Only recently, the molecular cloning of GDP-L-Fuc1:Asn-linked GlcNAc α1,6-fucosyltransferase (core α1,6-fucosyltransferase, Fuc-T C6, Fuc-T VIII) from porcine brain and from human gastric cancer cells has been reported (21, 22). As regards core α1,3-fucosyltransferase (Fuc-T C3), a first character

* This work was supported by Grant P 12528 from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: Fuc, L-Fucose; Fuc-T C3, GDP-L-Fuc:Asn-linked GlcNAc α1,3-fucosyltransferase (core α1,3-fucosyltransferase); Fuc-T C6, GDP-L-Fuc:Asn-linked GlcNAc α1,6-fucosyltransferase (core α1,6-fucosyltransferase); GnGn, GnGnF3, and GnGnF6, N-glycans, for structures, see Fig. 1; HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; GnGn, GnGnF3, GnGnF6, GnGnF8, MMF3, MMF8, GdGal, GalGnF3, GdGal, MM, M5Gn, and M5GnF8, N-glycans, for structures see Fig. 1, PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; Mes, 2-N-morpholinoethanesulfonic acid; bp, base pair(s).

This paper is available on line at http://www.jbc.org
terization of the enzyme from mung bean seedlings revealed its dependence on the presence of nonreducing terminal GlcNAc (23). In this paper, we report the purification to homogeneity of Fuc-T C3 from mung bean seedlings, the cloning of its cDNA by a PCR-based approach, and the expression of active recombinant Fuc-T C3 in baculovirus infected insect cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mung bean seedlings (germinated for 3 days in the dark) were kindly donated by Dr. Zun-Ho Wu (Vienna, Austria) and by Evergreen Co. (Oeynhausen, Austria). Activated CH-Sepharose 4B, S-Sepharose, and GDP-[-U-14C]fucose were obtained from Amersham Pharmacia Biotech. “GnGn-Sepharose” (see Fig. 1 for glycan structures) was prepared by coupling of GnGn-peptide (see below) to activated CH-Sepharose 4B according to the manufacturer’s instructions. GDP-[-fucose, bovine kidney N-acetyl-β-glucosaminidase, N-acetyl lactosamine (Galβ1–4GlcNAc), lacto-N-biose (Galβ1–3GlcNAc), lacto-N-tetraose (Galβ1–3GlcNAcβ1–3Galβ1–4Glc), EEF standard mixture, and IPL-41 medium were purchased from Sigma. GDP-hexanolamine-agarose was purchased from Calbiochem. Sequencing grade trypsin, N-glycosidase A, N-glycosidase F, alkaline phosphatase, PstI, and BamHI were from Roche Molecular Biochemicals. 2,5-Dihydroxybenzoic acid, Dabsylated GnGn-hexapeptide, were synthesized by Vienna Biocenter Genomics. The TA Cloning Kit was obtained from Invitrogen.

**Assay for Core α,1,3-Fucosyltransferase Activity**—Enzymatic activity of Fuc-T C3 was determined using GnGn-peptide and GDP-[-U-14C]fucose at substrate concentrations of 0.5 and 0.25 mM, respectively, in the presence of Mes-HCl buffer, Triton X-100, MnCl₂, GlcNAc, and AMP as described (25, 29). Where specified, other acceptors were used.

**Purification of Core α,1,3-Fucosyltransferase**—All purification steps were performed at 4 °C. Mung bean seedlings were homogenized with a kitchen blender using 0.75 volumes of extraction buffer per kg of beans. The extraction buffer consisted of 0.5 mM dithiothreitol, 1 mM EDTA, Triton X-100, and 50 mM Tris-HCl, pH 7.3, by stirring overnight. Subsequent centrifugation at 30,000 g for 40 min yielded the Triton X-100 extract which was further purified as follows.

Step 1: the Triton X-100 extract was applied to a column (5 × 28 cm) of DE52 cellulose (Whatman) previously equilibrated with buffer A (25 mM Tris-HCl buffer, pH 7.3, containing 0.1% Triton X-100 and 0.02% NaCl). The non-binding fraction was directly used for step 2.

Step 2: the sample was applied to a column (2.5 × 32 cm) of Affi-Gel Blue (Bio-Rad) equilibrated with buffer A. After washing the column with this buffer, adsorbed protein was eluted with buffer A containing 0.5 M NaCl.
Step 3: following dialysis of the eluate from step 2 against buffer B (25 mM sodium citrate buffer, pH 5.3, containing 0.1% Triton X-100 and 0.02% NaN₃) it was loaded onto a column (0.5 m containing 0.1 mol of each primer, 0.1 mM dNTPs, 2 mM MgCl₂, 10 mM Tris-HCl buffer of pH 9.0, 50 mM KCl, and 0.1% Triton X-100 and 0.02% NaN₃.) and subsequently loaded onto a column (0.5 m containing 0.1 mol of each primer, 0.1 mM dNTPs, 2 mM MgCl₂, 10 mM Tris-HCl buffer of pH 9.0, 50 mM KCl, and 0.1% Triton X-100 and 0.02% NaN₃.)

Step 4: the dialysed sample was applied to a column (0.5 × 4.5 cm) of GnGn-Sepharose previously equilibrated with buffer C. Elution of the bound protein was accomplished with buffer C containing 1 M NaCl instead of MnCl₂.

Step 5: the enzyme was then dialyzed against buffer D (25 mM Tris-HCl, pH 7.3, containing 10 mM MgCl₂, 0.1 mM NaCl, and 0.02% NaN₃) and subsequently loaded onto a column (0.5 × 4.5 cm) of GDP-Sepharose. Details are given under “Experimental Procedures.” Fractions from each column were assayed for protein (S₃) and subsequently loaded onto a column (0.5 m containing 0.1 mol of each primer, 0.1 mM dNTPs, 2 mM MgCl₂, 10 mM Tris-HCl buffer of pH 9.0, 50 mM KCl, and 0.1% Triton X-100 and 0.02% NaN₃.) and subsequently loaded onto a column (0.5 m containing 0.1 mol of each primer, 0.1 mM dNTPs, 2 mM MgCl₂, 10 mM Tris-HCl buffer of pH 9.0, 50 mM KCl, and 0.1% Triton X-100 and 0.02% NaN₃.)

The coding region of the putative Fuc-T C3 cDNA including the cytoplasmic and the transmembrane regions was amplified using the forward primer 5'-CTTTGCACTACTGAGGGCCGAA-3' and the reverse primer 5'-TCTGGACCACAATTGGAAT-3' or PCR was performed with an annealing temperature of 55 °C under conditions otherwise as described above. Both 5'- and 3'-RACE products were subcloned into pCR2.1 vector and sequenced.

**PCRs with Genomic DNA**—Genomic DNA was prepared out of lyophilized mung bean hypocotyls by means of the DNeasy Plant Kit (Qiagen) following the manufacturer’s instructions. PCRs were performed on 200 ng of DNA in 50 μl of solution containing 20 mmol of each of fucosyltransferase-specific primers (see below) essentially as described above except that the annealing temperature was raised to 58 °C. The three resulting PCR products (FSP34–59, FSP37–515, and FSP 32–511) were subcloned into pCR2.1 vector using the TA cloning Kit (Invitrogen) and sequenced. Forward primers 5'-GGACACCTACCATCACATAAC-3', 5'-ATGGCTGTGCCGTTGGATGTT-3', and 5'-CTGGAAGCTTCTGGTGCTG-3' and reverse primers 5'-CTTCAGCATATTCTGCTG-3', 5'-GAAAGGACGAAATGCTCTGATA-3', and 5'-GTACATTATTAGCGCAT-3' were used to cover cDNA regions from 317 to 522, 392 to 944, and 890 to 1550 bp, respectively.

**DNA Sequence Analysis**—Sequences of subcloned fragments were determined by the dyeodeoxynucleotide chain termination method using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM 310 Genetic analyzer (Perkin-Elmer). T7 and M13 forward primers were used for sequencing the PCR products cloned in pCR2.1. Sequencing of both strands of the complete coding region was performed by the Vienna VBC Genomics-Sequencing Service using the cycle sequencing method with infrared labeled primers (IRD700 and IRD800) and a LI-COR Long Read IR 4200 sequencer (Lincoln, NE).

**Expression of Reombinant Fuc-T C3 in Insect Cells**—The coding region of the putative Fuc-T C3 cDNA including the cytoplasmic and the transmembrane regions was amplified using the forward primer 5'-ccgggtactccagagatgatggtc-3' and the reverse primer 5'-ccggggactatgacttggat-3' and reverse primers 5'-cttcAGCATATTCTGCTG-3', 5'-GAAAGGACGAAATGCTCTGATA-3', and 5'-GTACATTATTAGCGCAT-3' were used to cover cDNA regions from 317 to 522, 392 to 944, and 890 to 1550 bp, respectively.

**Analysis of the Transferase Product**—Dabsylated GnGn-hexapeptide (2 nmol) was incubated with insect cell homogenate containing recombinant Fuc-T C3 (0.08 milliunit) in the presence of non-radioactive GDP-γ-fucose (10 nmol) under conditions otherwise identical to those described for determination of transferase activity (see above). A control experiment was performed with homogenate from mock-infected insect.
cells. After incubation for 16 h at 37 °C, aliquots of 0.5 μg were diluted 20-fold and analyzed by MALDI-TOF MS. In addition, aliquots of both samples were mixed to give similar concentrations of substrate and product. This mixture was diluted with 0.1 M ammonium acetate of pH 4.0 containing 10 microunits of β-glycosidase A or with 50 mM Tris/HCl of pH 8.5 containing 100 microunits (1 unit hydrolyzing 1 μmol of substrate/min) of N-glycosidase F, respectively. After 2 and 20 h, small aliquots of these mixtures were removed and analyzed by MALDI-TOF MS. The remaining half of the sample containing the Fuc-T C3 product was digested with N-glycosidase A. The resulting oligosaccharides were pyridylaminated and analyzed by reverse phase HPLC (8, 32, 33). The transferase product was degraded using N-acetyl-β-glucosaminidase and again analyzed by HPLC. Pyridylaminated GnGnF 6 derived from human IgG, MMF 8 from honeybee venom phospholipase A, GnGn, Gm, M, and MM from bovine fibrin were used as reference substances (23, 26, 34). Additionally, the mass of the pyridylaminated product was determined by MALDI-TOF MS (see below).

MALDI-TOF Mass Spectrometry—Mass spectrometry was performed on a DYNAMO (Thermo BioAnalysis, Santa Fe, NM), a linear MALDI-TOF MS capable of dynamic extraction (a synonym for delayed extraction). Two types of sample-matrix preparations were employed. Peptides and deaspartylated glycopeptides were dissolved in 5% formic acid and aliquots were spotted on the target, air dried, and overlaid with 1% tides and dabsylated glycopeptides were dissolved in 5% formic acid and aliquots were spotted on the target, air dried, and overlaid with 1%

| Purification step | Total protein | Total activity | Specific activity | Purification factor | Yield |
|------------------|--------------|---------------|------------------|-------------------|-------|
| Triton X-100 extract | 91,500 | 4,846 | 0.05 | 1 | 100 |
| DE52 | 43,700 | 4,750 | 0.10 | 2 | 98.0 |
| Affigel blue | 180.5 | 4,134 | 23 | 460 | 85.3 |
| S-Sepharose | 8.4 | 3,251 | 390 | 7,300 | 67.1 |
| GnGn-Sepharose | 0.13 | 1,944 | 8,030 | 160,000 | 21.5 |
| GDP-hexanolamine-Sepharose | 0.02 | 867 | 43,350 | 867,000 | 17.9 |

a A unit is defined as the amount of enzyme which transfers 1 μmol of fucose to acceptor per minute.

b Determined by amino acid analysis.

RESULTS

Purification of Core α1,3-Fucosyltransferase—Fuc-T C3 was purified from mung bean seedlings by Triton X-100 extraction of a crude microsomal preparation and several chromatographic steps including cation exchange and two types of affinity chromatography. The typical elution profile of activity from the S-Sepharose is shown in Fig. 2. Conveniently, this step provided separation from N-acetyl-β-glucosaminidase which otherwise would have degraded the ligand of the subsequent affinity chromatography on GnGn-Sepharose (Fig. 2). After the last purification step on GDP-hexanolamine-Sepharose, the final yield was 18 μg of protein from 5 kg of mung beans (Table I). SDS-PAGE revealed two bands, a major band at 54 kDa and one at 56 kDa (Fig. 3). In order to check whether the two polypeptides are distinct or just different forms of the same enzyme, the bands were compared by MALDI-TOF MS of tryptic peptides obtained in in-gel digestion. The mass spectra of the 54- and 56-kDa band were indistinguishable indicating that both bands represent the same enzyme, putatively Fuc-T C3.

Isoelectric focusing of purified Fuc-T C3 revealed several isoforms with pI values ranging from 6.8 to 8.2 (Fig. 3). The enzymatic activity of the different bands was verified by loading two lanes with 1 μg each of enzyme. One lane was silver stained, the other was used for measurement of enzymatic activity. For this purpose, the lane was cut into 4-mm pieces. After sonication in the presence of buffer A (see “Experimental Procedures”), Fuc-T C3 activity was determined in the supernatant. All gel pieces corresponding to stained bands gave high Fuc-T C3 activity, even the intensity of bands correlated with activity suggesting that all bands represented active transferase. The three major bands with pI values of approximately 6.8, 7.1, and 7.6 were accompanied by faint satellite bands. In two-dimensional electrophoresis, these satellite bands migrated slightly slower than the major species thus representing the 56-kDa band from normal SDS-PAGE (Fig. 3). Apparently, Fuc-T C3 occurs in at least 7 isoforms.

Partial Amino Acid Sequences and cDNA Cloning of Fuc-T C3—The major protein band of apparently 54 kDa was digested in-gel with trypsin. Four peptides were isolated by reverse phase HPLC and sequenced to yield the following peptide sequences: peptide 1, KPDAxFGLPQPSTAS; peptide 2, PETVYHIYHR; peptide 3, MESAEP-YAENNIA, and peptide 4, GRFAMESIYL. Attempts to sequence the N terminus of intact Fuc-T C3 failed. On the basis of peptides 1, 2, 3, and 3, degenerate oligonucleotides were synthesized and used as primers for reverse transcriptase-PCR as described under “Experimental Procedures.” The PCR products obtained with primers S1-A2 and S1-A3 consisted of 744 and 780 bp, respectively, both sharing the same 5′ end. The 780-bp fragment included the coding sequence for peptide 2. To obtain the full-length cDNA, 3′- and 5′-RACE was performed using the primers described under “Experimental Procedures.” Full-length cDNA consisted of 2.2 kilobases and contained an open reading frame of 1530 bp which included the coding region for all four peptides derived from the purified enzyme (Fig. 4).
Due to the lack of information about the natural N terminus of Fuc-T C3, the possible N terminus can only be deduced from potential initiation codons between the putative transmembrane region (see below) and the first stop codon toward the 5' end. The open reading frame that starts with the first Met residue located right beneath a stop codon encodes a protein of 510 amino acids, a molecular mass of 56.8 kDa, and a calculated pI of 7.51 (Fig. 4). A theoretical tryptic peptide map of the deduced complete amino acid sequence exhibited significant similarity to the map of purified Fuc-T C3 (data not shown).

Expression and Characterization of Recombinant Fuc-T C3—The coding region of Fuc-T C3 was engineered into a baculovirus transfer vector. Various amounts of progeny virus from the co-transfection were used to infect SF21 insect cells. In the best batch obtained, total fucosyltransferase activity of cells and supernatant was about 30 times higher than in the mock infected control batch. The endogeneous activity measured in the absence of recombinant transferase arises, however, essentially from insect Fuc-T C6 and only to a marginal extent from Fuc-T C3 (32, 36). Thus, the increase in Fuc-T C3 caused by the recombinant baculovirus is well above 100-fold.

Similar to the natural enzyme, the recombinant transferase displayed a broad maximum of activity around pH 7.0 when measured in Mes-HCl buffer and the presence of divalent cations, in particular of Mn2+1, enhanced its activity. Among the acceptors employed, GnGn-peptide gave the highest incorporation rates under standard assay conditions, closely followed by GnGnF6-peptide and M5Gn-Asn (Table II). The apparent \( K_m \) values for the acceptor substrates GnGn-peptide, GnGnF6-peptide, M5Gn-Asn, and for the donor substrate GDP-fucose were estimated to be 0.19, 0.13, 0.23, and 0.11 mM, respectively. No transfer was observed to MM-peptide which lacks the terminal GlcNAc residue on the 3-linked mannose regarded to be a structural requirement for core fucosyltransferases (1, 2, 37). By the standard assay, fucosyl transfer to GalGal-peptide could not be observed. However, a low rate of incorporation was demonstrated by MALDI-TOF MS (see later). Recombinant Fuc-T C3 was inactive toward common acceptors used for the determination of blood group \( \alpha_1,3/4 \)-fucosyltransferases which transfer fucose to GlcNAc residues at the nonreducing termini.
of oligosaccharides (Table II). Thus, with regard to substrate specificity and kinetic properties, the recombinant fucosyltransferase performed comparable to its natural counterpart (23).

Analysis of the Deduced Amino Acid Sequence of Fuc-T C3—

Analysis of Fuc-T C3 by “TMpred” (provided by EMBnet, Switzerland) suggested a transmembrane region between Asn-36 and Gly-54 which, remarkably, contains a glutamic acid residue (Fig. 5). The C-terminal, major part of the enzyme most likely comprises the catalytic domain and can therefore be assumed to face the lumen of the Golgi apparatus. Thus, mung bean Fuc-T C3 appears to be a type II transmembrane protein like all hitherto analyzed glycosyltransferases involved in glycoprotein biosynthesis (38).

A BLASTP search (with deactivated filter) of all data bases accessible via NCBI showed similarity of mung bean Fuc-T C3 to essentially all known mammalian, protozoan, and bacterial α,1,3/4-fucosyltransferases with probability values ranging from $7.4 \times 10^{-12}$ to $10^{-20}$ for a putative Vibrio cholerae fucosyltransferase. Remarkably, exon II alone was sufficient to retrieve by BLASTP all these α,1,3/4-fucosyltransferases with even higher probability values. In contrast, data bank searches with the individual exons I, III, and IV (see below) did not reveal a similarity to any known protein or nucleotide sequence. Alignments of mung bean Fuc-T C3 exon II with fucosyltransferases responsible for Lewis epitope synthesis revealed four regions of significant homology, (Fig. 6) as will be discussed later.

Exon-Intron Organization of Mung Bean Core α1,3-Fucosyltransferase—

Three fragments covering the whole open reading frame as overlapping pieces were amplified from genomic DNA by PCR. While the genomic PCR product covering cDNA region 392 to 944 bp had the size expected from cDNA, the fragments covering bp 174 to 522 and 890 to 1550 appeared considerably larger indicating them to contain introns. Therefore, these fragments were subcloned, sequenced, and the sequences were analyzed for potential donor and acceptor splice sites using NetPlantGene V2.0 (39). The suggested splice sites were between base pairs 384/385 (CAG g . . . cag G), 1049/1050 (AG gt . . . ag GT), and 1277/1278 (AG gt . . . ag GT) and agree with the cDNA sequence. Thus, the open reading frame of mung bean Fuc-T C3 gene is interrupted by three introns. The four exons therefore encode for amino acid residues 1–128, 129–350, 351–426, and 427–510.

Structural Characterization of the Fucosylated Product—

The structure of the product generated by the recombinant putative core α1,3-fucosyltransferase was analyzed in two ways. For both strategies, a dabsylated glycopeptide having the “GnGn” structure was used as acceptor. The sample (2 nmol) was incubated overnight with homogenate from baculovirus-infected S21 cells. An initial analysis by MALDI-MS revealed an almost complete fucosylation of the substrate in contrast to an approximately 5% conversion to fucosylated product in the mock infected control sample (data not shown). As was shown later by HPLC analysis of the pyridylaminated glycan, most of this product from endogenous insect cell fucosyltransferase was α1,6-fucosylated (see below).

The first analytical strategy to prove the identity of the product of the recombinant enzyme made use of the inability of N-glycosidase F to hydrolyze substrates with α1,3-fucose attached to the Asn-linked GlcNAc (27). Aliquots of the putative GnGnF-peptide were mixed with similar amounts of substrate (dabsyl-GnGn-peptide) and the mixtures were incubated with either N-glycosidase F or N-glycosidase A. The extent of hydrolysis was determined by MALDI-TOF MS after 2 and 20 h. Only N-glycosidase A was able to digest both substrate and fucosylated product. In contrast, N-glycosidase F, although having completely hydrolyzed the substrate ([M + H]+ = 2262.3) after only 2 h, did not hydrolyze the core α1,3-fucosylated glycopeptide ([M + H]+ = 2408.4) even after 20 h of incubation.

As a second proof, the remaining 1 nmol of Fuc-T C3 product in sample A was digested with N-glycosidase A and pyridylaminated and the fluorescent derivatives were subjected to reverse phase HPLC. The significant reduction of elution time of the product compared with the substrate implies α1,3-fucosylation.
of the reducing terminal GlcNAc (Fig. 7) (4, 36, 40). No other known structural feature of N-linked oligosaccharides exerts such a strong and characteristic effect on the retention time of pyridylamino glycans (40). The compounds mass of 1564.5 agreed exactly with the mass expected for the sodium adduct of the pyridylamino glycan. To exclude any possibility of fucose being linked to a nonreducing terminal GlcNAc and to allow comparison of retention time with a reference oligosaccharide analyzed previously by independent methods, i.e., pyridylamino MMF3 from honeybee venom phospholipase (Fig. 1) (26), the product was digested with N-acetyl-b-glucosaminidase. Indeed, the putative MMF3 coeluted with the reference glycan.

**Fig. 6.** Conserved regions of core and blood group α1,3-fucosyltransferases. Four regions of apparent homology between mung bean Fuc-T C3 and most currently known α1,3/4-fucosyltransferases are shown. Conserved residues are represented by white letters on black background or, if common to only a few transferases, on gray background. The blocks B and D represent the highly conserved regions I and II previously described (43, 44). In the case of the putative fucosyltransferase from Dictyostelium discoides and the EST from S. japonicum, the gene products have not yet been analyzed. A lysine residue shown to be essential for activity of human Fuc-T V and VII is marked by an arrow (45). The number of residues between the depicted partial sequences are given in brackets. Transferases are identified by SwissProt (square brackets) or, if not applicable, by GenBank accession numbers.

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The results from both experiments provide convincing evidence that the recombinant enzyme transfers fucose in α1,3-linkage to the reducing terminal GlcNAc of a complex N-glycan, thus being a core α1,3-fucosyltransferase.

The above described analytical strategy was applied to investigate fucosyl transfer to β1,4-di- and monogalactosylated N-glycans. By MALDI-TOF MS, the transfer rate to dabsylated GalGal-peptide was found to be about 0.7% of that with dabsylated GnGn-peptide. However, when a mixture of GnGn-, GalGn-, GalGal-, and GalGal-peptide (prepared by partial enzymatic degalactosylation of dabsylated GalGal-peptide) was used as the substrate, about half of the monogalactosylated species were readily fucosylated just as the GnGn-peptide (Fig. 8, upper panel). To determine which of the two monogalactosylated isomers had been fucosylated, the oligosaccharides were analyzed by HPLC. To allow comparison of elution times with reference oligosaccharides, the mixture was digested with N-acetyl-β-glucosaminidase and, after heat denaturation of this enzyme, with β-galactosidase. The fact that the positions of core fucose as well as of terminal GlcNAc residues strongly influence the elution positions of pyridylaminated N-glycans on reverse phase (4, 32, 34, 40) allowed to identify the four oligosaccharides obtained by the above described procedure (Fig. 8, lower panel). The glycans MMF<sup>3</sup>, GnMF<sup>3</sup>, MgN, and GnGn are regarded as the glycosidase digestion products of GnGnF<sup>3</sup>, GalGnF<sup>3</sup>, GnGn, and GalGal, respectively. Thus, Fuc-T C3 had acted on β1,4-monomethylated N-glycans if Gal was located on the 6-arm (GalGn) but not if it was on the 3-arm (GnGn).

**FIG. 7.** HPLC analysis of pyridylaminated fucosyltransferase product. Glycopeptides obtained by incubation of dabsylated substrate (GnGn-peptide) with insect cell homogenates were digested with N-glycosidase A and the oligosaccharides were pyridylaminated and subjected to reverse phase HPLC. A, in the control experiment with mock-infected insect cells, a small peak which, according to its elution time and to previous work (36), represents GnGnF<sup>3</sup>, can be seen in addition to residual substrate; B, in the sample prepared with recombinant enzyme, almost the entire substrate was converted to a product (P) which exhibits the very low retention indicative of core α1,3-fucosylation; C, isolated transferase product, putative GnGnF<sup>3</sup>; D, transferase product after digestion with N-acetyl-β-glucosaminidase; E, MMF<sup>3</sup> from honeybee phospholipase A2.

**FIG. 8.** Fucosyltransfer to β1,4-mono- and digalactosylated substrate. A mixture of dabsylated glycopeptides having the structures GnGn, GalGn, GnGal, and GalGal was prepared by limited digestion with β-galactosidase and used as the substrate for Fuc-T C3. After 20 h, the transferase reaction mixture was analyzed by MALDI-TOF MS (upper panel). All of the GnGn was converted to GnGnF<sup>3</sup> (peak 1: calculated [M + H]<sup>+</sup> = 2408.4), whereas only about half of the isobaric pair GalGn/GnGal (peak 2, 2424.5) gave rise to a fucosylated product (peak 3, 2570.6), and only a minute amount of GalGal (peak 4, 2586.6) had been fucosylated (peak 5, 2732.8). Other peaks in the spectrum mainly represent sodium adducts. While the structure of the fucosylated GalGal (5) has not been analyzed, the monogalactosylated and fucosylated glycan was assigned the structure GalGnF<sup>3</sup> (see Fig. 1) by reverse phase HPLC of the pyridylaminated oligosaccharides which had been sequentially digested with N-acetyl-β-glucosaminidase and β-galactosidase. Arrows indicate the elution positions of MGnF<sup>3</sup> (A), MMF<sup>3</sup> (B), GnGnF<sup>3</sup> (C), GnMF<sup>3</sup> (D), MGn (E), MM (F), GnGn (G), and GnM (H). Thus, in the original mixture of glycopeptides, only those with a terminal GlcNAc on the 3-arm had been fucosylated.

**DISCUSSION**

As the first enzyme of its kind, mung bean core α1,3-fucosyltransferase has been purified, cloned, and heterologously expressed. Partly based on previous work (23), a purification scheme was established which gave an almost million-fold purification. Nevertheless, SDS-PAGE revealed two bands of apparently 54 and 56 kDa. Mass spectrometric peptide mapping indicated these bands to represent isoforms of the same enzyme. Zeng et al. (41) who observed a similar pattern for soybean xylosyltransferase suggested limited proteolysis as an explanation. Different glycosylation site occupancy may likewise account for the small mass difference. Whatever their difference might be, the ratio of the two isoforms differed dra-
glycosidase to act on core a N-recombinant enzyme rendered a glycopeptide resistant against galactosyltransferase (42). Incorporation of fucose by the potential acceptor for fucose. Remarkably, GnGnF6 which cer-
time that the biosynthetic intermediate M5Gn is shown to be a peptide with similar efficiency. It is, to our knowledge, the first e.g. part (residues 351 to 384) can be tentatively aligned with, and Cys-
markably, bacterial and protozoan fucosyltransferases exhibit
tors which are derived from mammalian glycoproteins. These
be noted, that no sequence similarities of residues 385–510
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