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A PLANT FUCOSYLTRANSFERASE WITH HUMAN LEWIS BLOOD-GROUP SPECIFICITY

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ABSTRACT

A fucosyltransferase from mung bean seedling was found to transfer L-fucose from GDP-fucose to the Type 1 disaccharide β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-OR [R = $(CH_2)_8COOMe$]. The product, which was detected by an anti-Le^a antibody in a novel ELISA assay, was isolated and shown to be the human Le^a blood-group determinant β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-OR by ^{1}H-n.m.r. spectroscopy. This enzyme activity is distinct from that of the human Lewis-fucosyltransferase since α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-OR is a very poor substrate, while the Type 2 disaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-OR is not an acceptor. In common with the Lewis fucosyltransferase, the H-Type 1 trisaccharide α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-OR was an excellent substrate for the enzyme. This new enzyme activity was further characterized with respect to pH, nucleotide, Mn^{2+} dependence, and acceptor specificity against a panel of synthetic oligosaccharides.

INTRODUCTION

Important changes in the structures of cell-surface oligosaccharides are now known to accompany both normal and abnormal cellular development where new carbohydrate structures appear as stage-specific differentiation markers or as tumor-associated antigens[1–3]. Many of the structures involved contain fucosylated and/or sialylated Type 1 [β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc] and Type 2 [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc] sequences which can be carried on glycolipids as well as on both *O*- and *N*-linked glycoproteins. These structures appear as a result of the

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aberrant glycosylation that accompanies such cancers as human colorectal, gastric, and pancreatic carcinomas[4]. The functional significance of aberrant glycosylation is not yet understood.

The availability of complex oligosaccharides of well-defined structure, and in sufficient quantity, is critical to the study of the function of cell-surface carbohydrates. Such oligosaccharides are invaluable as inhibitors of cell–cell and cell–matrix adhesion, in the production and screening of monoclonal antibodies, and in the identification and characterization of carbohydrate-specific receptors. Many of the structures of interest, however, occur as minor components of the complex mixture of molecules extracted from biological sources and their large-scale purification can be prohibitively difficult. Our approach to the provision of such oligosaccharides is now shifting from total chemical synthesis to combined chemical/enzymic syntheses. The merit of this approach is that it requires the chemical synthesis of only fairly small oligosaccharides, usually 2–5 sugar residues in size, which can then be enzymically homologated to a diversity of larger and more complex structures. The success of this strategy hinges on ready access to glycosyltransferases with well-characterized acceptor specificities.

In the course of surveying animal tissues for fucosyltransferase activities which would act on Type 1 and Type 2 sequences, we became aware of the work of Szumilo *et al.*[5] who reported that the activity of *N*-acetylglucosaminyltransferases I and II, two key glycosyltransferases involved in the biosynthesis of complex *N*-linked carbohydrates, could be readily detected in the extracts of mung bean seedlings. We now report that mung bean seedlings also contain a fucosyltransferase activity which acts on the Type 1 disaccharide sequence to produce β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc, the human Le^a blood-group determinant, while the Type 2 disaccharide is not an acceptor. This fucosyltransferase represents an activity different from that of the human enzyme, since 2′-fucosyl-lactose was found to be a very poor substrate[6,7]. The enzyme should therefore be of significant value in the preparation of oligosaccharides with structurally defined carbohydrate sequences. Part of this work has been presented in preliminary form[8].

RESULTS AND DISCUSSION

Hydrophobic synthetic acceptor oligosaccharides are ideal as substrates in the radioactive assay of glycosyltransferases since the enzymically produced, labelled products can be rapidly isolated and quantitated using reverse-phase C_{18} cartridges[9]. Using the Type 1 [β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-OR, **1**] and Type 2 [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-OR, **2**] disaccharides, as their hydrophobic 8-methoxycarbonyloctyl glycosides [R = $(CH_2)_8COOMe$], mung bean seedlings were examined for fucosyltransferase activity using this assay. The Triton X-100 extract of a seedling microsomal fraction was thus found to transfer fucose from GDP-[^{14}C]fucose to the Type 1 acceptor (**1**) but not to the isomeric Type 2 structure (**2**).

The only plant fucosyltransferase activities of which we are aware catalyze the fucosylation of the 3-position of asparagine-linked chitobiose[10] or transfer to cell-wall galactosylated xyloglucans[11]. The extract showed no activity when assayed using β-D-Gal*p*-OR as acceptor[9], which suggested, by analogy with mammalian glycosyltransferases[12], that the activity was not a (1→2)-α-fucosyltransferase.

Evidence for the identity of the product of the fucosylation of **1** was obtained using an enzyme-linked immunosorbent assay (ELISA). This assay took advantage of the availability of the anti-Le^a monoclonal antibody CF4-C4 whose combining site has been exhaustively probed using synthetic oligosaccharide structures[13,14]. This antibody has an absolute requirement for the α-L-fucopyranosyl unit in the Le^a trisaccharide **3** and, consequently, shows no cross-reaction with the Type 1 disaccharide (**1**). Incubation of mung bean extract and unlabelled GDP-fucose in microtiter plates coated with a synthetic glyconjugate[15] of **1** produced a product which was bound by antibody CF4-C4. This observation strongly suggested the conversion of **1** into the Le^a-trisaccharide **3**.

The unequivocal proof of structure of the enzymically produced trisaccharide was obtained through a preparative incubation of the extract with unlabelled GDP-fucose and **1**. The product was isolated by adsorption onto a C_{18} cartridge, prepara-

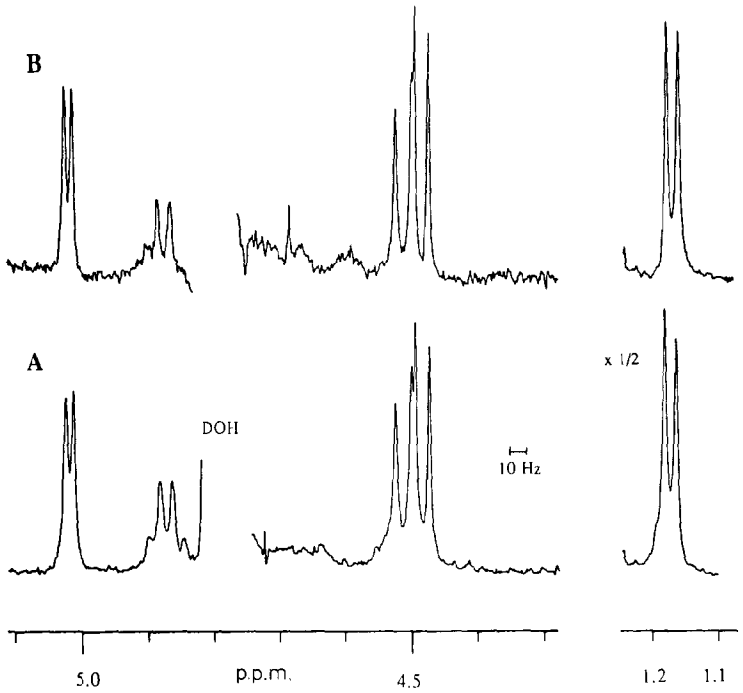

Fig. 1. Partial 360-MHz ^{1}H-n.m.r. spectra of A, chemically synthesized[15] Le^a trisaccharide **3**; and B, the product of the action of the fucosyltransferase preparation on the Type 1 disaccharide **1**. Characteristic signals for the α-L-Fuc*p* residue were observed at δ 5.021 (H-1″, $J_{1'',2''}$ 4 Hz), 4.874 (H-5″, $J_{5'',6''}$ 6.5 Hz), 1.178 (H-6″). The rest of the spectra were identical.

tive t.l.c. on silica gel, and h.p.l.c. on a PAC-column. The partial ^{1}H-n.m.r. spectrum of the product is shown in Fig. 1 where it is seen to be identical to that of the 8-methoxycarbonyloctyl Le^a trisaccharide, β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-OR (**3**), which was available from a previous total chemical synthesis[15]. The product is therefore definitively established to be that which would be produced by the action of the Lewis-fucosyltransferase on **1**.

Manganese was found to be a non-essential activator. The activity of the crude extract was doubled by the addition of 5mM Mn^{2+}. Beyond this concentration, activity decreased gradually, returning to original levels at 40mM and <10% at 100mM. This behaviour is similar to that reported for the human milk Lewis fucosyltransferase[6]. The activity could not be reduced by exhaustive dialysis against 0.1mM EDTA, and was constant in the pH range 6.0–8.7.

Preincubation of the enzyme extract with GDP-fucose for 45 min prior to the addition of acceptor **1** almost completely (>95%) abolished the apparent activity, suggesting the presence of a GDP-fucose hydrolyzing activity. Neither ATP, CTP, ADP, GDP, or GMP were good hydrolase inhibitors and, in addition, inhibited the fucosyltransferase activity by up to 60% at 8mM. UMP, CMP, and UDP were, however, effective hydrolase inhibitors and, consequently, kinetic parameters were measured in the presence of 2mM UMP.

The K_m for GDP-fucose was 7.8 μM (Fig. 2) at 1mM (6 × K_m) acceptor **1**, and 5mM $MnCl_2$ with a V_{max} of 144 pmol/min/mg of protein. For kinetic studies in the absence of added $MnCl_2$, the K_m for GDP-fucose (6.3μM) was essentially unchanged, while V_{max} decreased to 77 pmol/min/mg.

The acceptor specificity of the fucosyltransferase was examined using the panel of synthetic acceptors listed in Table I. The enzyme fucosylates both the Type 1 sequence (**1**) to produce the Le^a trisaccharide **3**, and the H-Type 1 trisaccharide **4**, presumably producing the Le^b tetrasaccharide determinant α-L-Fuc*p*-

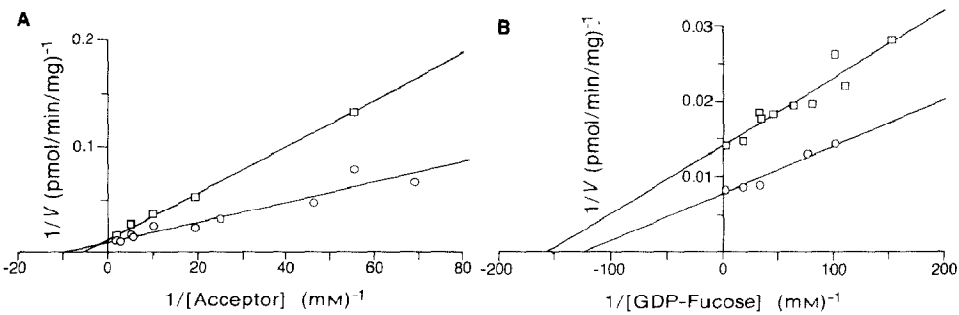

Fig. 2. A, Double reciprocal plots for Type 1 acceptor (**1**) as substrate, with (○) and without (□) 5mM $MnCl_2$. Incubations were performed at 37° for 30–120 min and contained from 9–500μM compound **1**, 0.014 or 0.028 μCi of GDP-[U-^{14}C]fucose, 100μM GDP-fucose, and 46.5 μg of microsomal protein in 25 μL of Tris-HCl extraction buffer with 2mM UMP. B, Double reciprocal plots for GDP-fucose with (○) and without (□) 5mM $MnCl_2$. Incubations were performed at 37° for 30–120 min, and contained from 7–789μM GDP-fucose, 0.016 μCi of GDP-[U-^{14}C]fucose, 25.3 nmol of the Type 1 acceptor (**1**), and 4.65 μg of microsomal protein in a final volume of 25 μL of 50mM Tris-HCl extraction buffer with 2mM UMP.

TABLE I

ACCEPTOR SUBSTRATE SPECIFICITY OF MUNG BEAN FUCOSYLTRANSFERASE

Acceptor structure	*Relative rate (%)*	*Apparent* K_m *(μM)*	V_{max} *(pmol/min/mg)*
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-OR (**1**)	100[a]	160 170[b]	144 77[b]
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-OR (**4**)	64.7	22[b]	45[b]
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-OR (**2**)	<1	—	—
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-OR (**5**)	4.8	—	—
α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-OR (**6**)	5.8	—	—
α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-OR (**7**)	7.7	—	—
α-D-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-OR (**8**)	<1	—	—

[a]100% = 133 pmol/min/mg of protein. [b]Assayed in the absence of added $MnCl_2$.

(1→2)-β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-OR. Surprisingly, the H-Type 2 trisaccharide (**6**) showed weak acceptor activity under conditions where fucosylation of the Type 2 disaccharide (**2**) could not be detected. The product of this reaction was not characterized and it therefore remains to be established whether the same or a different fucosyltransferase activity was being detected. The 2′-fucosyl-lactose trisaccharide **5** showed only poor acceptor activity. The enzyme also transfers to the sialylated Type 1 structure (**7**), presumably producing the sialyl-Le[a] determinant, while the sialylated Type 2 trisaccharide (**8**) is not an acceptor.

The Lewis fucosyltransferase has been extensively (>500,000-fold) purified from human milk[6,7]. It has generally been regarded as an α-3/4-fucosyltransferase acting on both Type 1 and Type 2 chains, as well as on 2′-fucosyl-lactose. Recently, however, it has been reported[16] that the α-3 activity measured using *N*-acetyl-lactosamine as substrate can be greatly decreased by further purification. The resulting fucosyltransferase preparation had similar activity against both the Type 1 disaccharide and 2′-fucosyl-lactose, but the relative activity against *N*-acetyl-lactosamine was decreased to 10%. The substrate specificity of the plant enzyme presented in Table I clearly indicates it to be distinct from the human Lewis enzyme, since there is no detectable activity against the Type 2 disaccharide **2** and the 2′-fucosyl-lactose structure **5** is a very poor substrate.

The effect of Mn^{2+} on the kinetics of fucosylation of **1** is also summarized in Table I and Fig. 2. As seen in the kinetic studies with GDP-fucose, added Mn^{2+} has little effect on the K_m for the acceptor substrate but rather increases the rate of reaction through an increase in V_{max}. Preliminary work also demonstrated the existence of this enzyme activity in pea seedlings. Further characterization and comparisons with the human Lewis enzyme await the purification (work in progress).

EXPERIMENTAL

General. — Protein concentrations were estimated using the Bradford assay[17], with bovine serum albumin (BSA) as the standard, after removal of detergent by a 15-min batch-adsorption using Bio-Beads SM2. ^{1}H-N.m.r. spectra were recorded with a Bruker WM-360 instrument for solutions in deuterium oxide containing acetone (0.01% v/v, δ 2.225) as internal standard at 23°. ELISA plates were read on a Bio-Tek EL-309 automated EIA plate reader. Phosphate-buffered saline solution (PBS) was a solution of pH 7.4 containing 7.8mM Na_2HPO_4, 2.2mM KH_2PO_4, 0.9% of NaCl, and 15mM NaN_3; PBST was prepared by adding 0.05% Tween 20 to PBS.

Materials. — Compounds **1** and **3**[15], **2** and **6**[18], **4**[19], **5** (R. M. Ratcliffe, unpublished), **7** and **8**[20], GDP-fucose[21] were available from previous work. GDP-L-[U-^{14}C]fucose (248 mCi/mmol) was from New England Nuclear, Sep-Pak C_{18} cartridges from Waters Associates, Triton X-100 from Calbiochem, and Bio-Beads SM2 and a protein assay kit from Bio-Rad. Preparative t.l.c. plates (PLK5F) and a Partisil 5 PAC column were from Whatman. Jumbo mung-bean seeds (*Phaseolus aureus*) from Bima Industries Inc. were obtained from a local health-food store. Microtiter plates coated with synthetic β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc glycoconjugate, product number 67-811P (Plate Le^c), were from Chembiomed Ltd. Sugar nucleotides, alkaline phosphatase-conjugated goat anti-mouse IgG (whole molecule), and 5-mg *p*-nitrophenyl phosphate tablets were from Sigma. Antibody CF4-C4 was affinity-purified as previously described[14].

Fucosyltransferase assays. — The enzyme preparation used for both kinetics and preparative synthesis was a microsomal fraction from mung bean seedlings prepared according to the method of Szumilo *et al.*[5] and extracted into 50mM Tris-HCl buffer (pH 7.2) containing 1% of Triton X-100, 20% of glycerol, 1.6mM NaN_3, 1mM NaCl, and 5μM dithiothreitol (extraction buffer).

Fucosyltransferase activity was quantitated using a modified Sep-Pak assay[9]. Incubation mixtures for acceptor specificity studies contained 50μM GDP-fucose, 36,000 d.p.m. GDP-L-[U-^{14}C]fucose, 2mM UMP, 5.5mM $MnCl_2$, 1mM acceptor, and enzyme (47 μg of protein) in a total volume of 25 μL of extraction buffer. After incubation at 37° for 60 min, unreacted GDP-fucose was removed by loading the reaction mixture onto a Sep-Pak C_{18} cartridge, and washing with H_2O (5 × 5 mL) to background counts. Radiolabelled products were eluted in methanol (2 × 5 mL), and quantitated as d.p.m. in 10 mL of ACS scintillation cocktail with a Beckman LS1801 scintillation counter.

For synthetic preparative reactions, mixtures contained 2 mL of plant extract (3.7 mg of protein), 1.55mM GDP-fucose, 180,000 d.p.m. GDP-L-[U-^{14}C)fucose, 1mM β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-OR (**1**), and 1mM NaN_3. These incubations were carried out for 24 h at 37°, after which the radiolabelled products were isolated as described above. Additional purification of the product from unreacted acceptor and Triton X-100 was achieved by preparative t.l.c. on silica gel (MeOH–CH_2Cl_2–

H_2O 30:65:8, R_F 0.7) and h.p.l.c. on a PAC column. The mobile phase was CH_3CN–H_2O (90:10) at a flow rate of 2.5 mL/min and elution of the product (30% yield; retention time, 11 min) was monitored at 214 nm.

The pH stability and relative activity were established by dialyzing plant extracts into 50mM sodium cacodylate buffer (pH 6.0–6.9) or Tris-HCl (pH 6.9–8.7) buffers which also contained all of the components of the extraction buffer. Activity was monitored as for acceptor specificity trials, using 0.5mM acceptor and 0.1mM GDP-fucose in a total volume of 25 μL.

ELISA assays. — The fucosyltransferase preparations, 50 μL, were made 13.3μM in GDP-fucose and added in duplicate to the wells of a Plate-Le^c microtiter plate which is supplied coated with β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc glycoconjugate[15]. Control wells contained 13.3μM GDP-fucose in extraction buffer or fucosyltransferase preparation without added GDP-fucose. After incubation for 14 h at ambient temperature, the solutions were aspirated and the wells were washed with 2 × 300 μL of H_2O, and 2 × 200 μL of PBS. Antibody CF4-C4, 0.39 μg/mL in PBST containing 1% of BSA (100 μL), was added to each well, and the plate was kept for 2 h at ambient temperature when the antibody solution was removed by aspiration. After washing the well with PBS (3 × 200 μL), alkaline phosphatase-conjugated IgG (100 μL) diluted 1/650 fold with 1% BSA in PBST was added. After 2 h, the solution was removed by aspiration and the wells were washed with PBS (3 × 200 μL). *p*-Nitrophenyl phosphate substrate (100 μL), prepared by dissolving a 5-mg tablet in 5 mL of 1M diethanolamine–HCl buffer (pH 9.8), containing 1% of BSA and 500μM $MgCl_2$, was added to each well. The formation of *p*-nitrophenoxide was monitored by the increase in absorbance at 405 nm. The absorbance in the control wells was 0.13 after 24 min and 0.25 after 45 min. With both plant extract and sugar nucleotide, the average absorbances for these times were 0.65 and 1.4, demonstrating that synthesis of the Le^a determinant had occurred in these wells.

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