Purification and cDNA Cloning of Porcine Brain
GDP-1-Fuc:N-Acetyl-β-D-Glucosaminide α1→6Fucosyltransferase*

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GDP-L-Fuc:N-acetyl-β-D-galactosaminide α1→6fucosyltransferase (α1→6Fuct; EC 2.4.1.68), which catalyzes the transfer of fucose from GDP-Fuc to N-linked type complex glycopeptides, was purified from a Triton X-100 extract of porcine brain microsomes. The purification procedures included sequential affinity chromatographies on GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→2)Manβ1→4GlcNAcβ1→4GlcNAc-Asn-Sepharose 4B and synthetic GDP-hexanolamine-Sepharose 4B columns. The enzyme was recovered in a 12% final yield with a 440,000-fold increase in specific activity. SDS-polyacrylamide gel electrophoresis of the purified enzyme gave a major band corresponding to an apparent molecular mass of 58 kDa. The α1→6Fuct has 575 amino acids and no putative N-glycosylation sites. The cDNA was cloned in to pSVK3 and was then transiently transfected into COS-1 cells. α1→6Fuct activity was found to be high in the transfected cells, as compared with non- or mock-transfected cells. Northern blotting analyses of rat adult tissues showed that α1→6Fuct was highly expressed in brain. No sequence homology was found with other previously cloned fucosyltransferases, but the enzyme appears to be a type II transmembrane protein like the other glycosyltransferases.

It has been reported that the structures of glycopeptides change during the development and differentiation of embryos (1–4). Detailed analysis of specific antigens on the surface of various carcinoma cells revealed that carcinoma-specific sugar chains are expressed on the cell surface. A well documented phenotypic alteration of these specific sugar chains is the increase in the molecular weight of cell surface complex type N-linked glycan in transformed cells. This change has been observed regardless of the nature of the transforming agent: oncogenic viruses (5–9), chemical mutagens (10–11), or DNA from unrelated tumor cells (12–14). This phenomenon was thought to reflect the deviation of carcinoma cells from the ordinary differentiation processes. α-Fucose residue attached to asparagine-linked GlcNAc also have some relationship with carcinogenesis. A difference in the binding pattern of serum α-fetoprotein with lentil lectin between hepatocellular carcinomas and benign liver diseases has been reported (15–17). Analyses of the carbohydrate structure of α-fetoprotein from hepatocellular carcinoma cell lines have indicated that almost all of the carbohydrates of α-fetoprotein are α1→6-fucosylated (18). α-Fetoprotein produced by germ cell tumors, such as yolk sac tumors, is also highly fucosylated (19). The activity of α1→6Fuct1 was higher in hepatocellular carcinoma tissue than in non-tumor tissue (20) and was induced by the transfection of the ras protooncogene into 3T3 fibroblast cells (21).

Schacter et al. (22, 23) first characterized α1→6Fuct in porcine liver using a partially purified enzyme extract. The special release of α1→6Fuct from platelets during blood clotting has been reported (24, 25), alteration of fucosylation has been reported in cystic fibrosis glycoproteins from different sources, and α1→6Fuct from human fibroblasts of cystic fibrosis patients has been purified and characterized (26, 27). But little is known about this enzyme, and the α1→6Fuct gene has not yet been cloned. In this study we purified a novel α1→6Fuct, which is a different enzyme from the α1→6Fucts previously reported, especially in terms of the pH optimum and molecular weight. The cDNA sequence of this enzyme was also determined for the first time. A highly specific assay method involving a fluorescent reagent, 4-(2-pyridylamino)butylamine (PABA), made it possible to purify the enzyme (28).

EXPERIMENTAL PROCEDURES

2-Amino pyridine was obtained from Wako Pure Chemicals. β-Galactosidase (Aspergillus sp.) was obtained from Toyobo Co. TSK-gel ODS-80TM and Amide-80 columns were purchased from Tosoh. Bovine γ-globulin and Pronase (Streptomyces griseus) were purchased from Sigma and Seikagaku Kogyo Co., respectively.

Methods
Preparation of Substrate for α1→6Fuct
Fluorescence-labeled oligosaccharides were obtained from the corresponding oligosaccharides as described previously (28). Briefly, a glycopeptide derived from bovine γ-globulin on digestion with Pronase was coupled with PABA using water-soluble carbodiimide. Further purification was performed by HPLC on a TSK-gel ODS-80TM column (4.6 × 150 mm) that had been equilibrated with 20 mM acetate buffer, pH 4.0, containing 0.1% butanol, at a flow rate of 1 ml/min isocratically. The eluted pyridylamino sugars were detected with a fluorescence spectrophotometer, the excitation and emission wavelengths being 320 and 400 nm, respectively.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D86723.

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1 The abbreviations used are: α1→6Fuct, GDP-L-Fuc:N-acetyl-β-D-glucosaminide α1→6fucosyltransferase; PABA, 4-(2-pyridylamino)butylamine; GDP-Fuc, guanosine diphosphofucopyranoside; HPLC, high performance liquid chromatography; GnGnF-bi-, GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→2)Manβ1→4GlcNAcβ1→4GlcNAc-; GnGnF-tri-, GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→2)Manβ1→4GlcNAcβ1→4GlcNAc-; 4-(Fucol)-1→6GlcNAc-; MES, 2-(N-morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
The enzyme activity was determined as described (28). In brief, the standard incubation mixture for the 1–6FucT assay contained the following components, in a final volume of 50 μl: 200 mm MES-NaOH buffer, pH 7.0, 0.1% Triton X-100, 50 μM acceptor (GnGn-Asn-PARA), and 500 μM donor (GDP-4-Fuc) substrates. After the mixture had been incubated at 37 °C for 2 h, the reaction was stopped by heating at 100 °C for 1 min. The sample was centrifuged at 15,000 × g for 10 min, and then 10 μl of the supernatant was used for analyses. The product was separated by HPLC on a TSK-gel ODS-80TM column (4.6 × 150 mm). Elution was performed at 55 °C with 20 mM acetate buffer, pH 4.0, containing 0.1% butanol in an isocratic way. Fluorescence of the column eluate was detected with a fluorescence spectrophotometer (model RF 535; Shimadzu, Japan), the excitation and emission wavelengths being 320 and 400 nm, respectively. The amount of product was estimated from the fluorescence intensity. The specific activity of the enzyme was expressed as pmol of fucose transferred/h/mg of protein. Protein was determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

Preparation of GDP-Hexanolamine

GDP-hexanolamine was synthesized by the method of Nunez, et al. (29) with a slight modification. Briefly, GMP was activated with morphophosphate and then coupled to 6-aminohexylphosphate. The end product was completely purified on a TSK gel ODS TM80 column (4.6 × 150 mm). The structure of the GDP-hexanolamine column was confirmed by 1H NMR analyses (data not shown).

Purification and Column Chromatography of 1–6FucT

Step 1—A porcine brain (100 g) was homogenized in 4 volumes of 10 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 1 mM benzamidine hydrochloride, and 20 μM 4-(amidinophenyl)methanesulfonyl fluoride, with a Polytron homogenizer (Brinkmann Instruments).

Step 2—After centrifugation at 900 × g for 10 min, the resultant supernatant was centrifuged at 105,000 × g for 1 h. The pellet was resuspended in a 1000-mL solution of 20 mM phosphate-KOH, pH 7.0, 50 mM KCl, 5 mM EDTA, and 0.05% Triton X-100 buffer. The end product was completely purified on a TSK-gel ODS-80TM column (4.6 × 150 mm). The structure of the GDP-hexanolamine column was confirmed by 1H NMR analyses (data not shown).

| Tissues       | Specific activity pmol/h/mg protein |
|---------------|-----------------------------------|
| Brain (3)     | 733.0 ± 42.0                      |
| Spleen (3)    | 192.0 ± 21.0                      |
| Testis (3)    | 149.0 ± 4.7                       |
| Stomach (2)   | 59.4 ± 9.4                        |
| Lung (3)      | 45.3 ± 9.1                        |
| Kidney (3)    | 21.4 ± 3.1                        |

The values in parentheses indicate the number of specimens examined. Each value represents the mean ± S.D.

| Steps          | Volume | Protein | Total activity | Specific activity | Purification | Yield % |
|----------------|--------|---------|----------------|------------------|--------------|---------|
| Triton X-100 extract | 75     | 3,680   | 2,220          | 0.6              | 1            | 100     |
| GnGn-bi-Asn-Sepharose 4B | 10     | 0.80    | 1,350          | 1,688            | 2,813        | 61      |
| GDP-CH2-6NH-Sepharose 4B | 0.2    | 0.01    | 262            | 262,200          | 437,000      | 12      |

Determination of Partial Amino Acid Sequences of 1–6FucT

To determine the N-terminal amino acid sequences, the material corresponding to the 54-kDa band was excised from a polyvinylidine difluoride membrane (Millipore) and then sequenced with an Applied Biosystems 473A Protein Sequencer. To determine internal amino acid sequences, 13 μg of the purified protein transferred onto a polyvinylidene difluoride membrane was digested with 1 μg of lysyl endopeptidase in 100 mM Tris and 5% acetonitrile, pH 8.2, at 37 °C for 2 h. The resultant peptides were separated by reverse phase HPLC on a Vydac C-18 column (250 × 2.1 mm), using a 0–60% acetonitrile gradient in 0.05% trifluoroacetic acid. Five peptides were obtained and applied to a Poros-traps, a non-bonded silica-based, pressurized glass filter for amino acid sequencing and were analyzed with an Applied Biosystems 473A protein sequencer.

Construction of the cDNA Encoding 1–6FucT cDNA

Total RNA was isolated from a porcine brain according to general methods. Poly(A)⁺ was further purified on an oligo(dT)-cellulose (Pharmacia) column. The first strand cDNA was synthesized with a cDNA synthesis kit (Life Technologies, Inc.), using primer A11, according to the manufacturer’s manual (see Fig. 8).

Polymerase Chain Reaction (PCR)

Four oligonucleotides were synthesized for use as primers in PCR (see Fig. 7). PCR was carried out in 50-μL reaction mixtures containing first strand cDNA corresponding to 1 μg of poly(A)⁺ RNA, 50 pmol of a pair of degenerate oligonucleotide, 50 μM dNTP, 5 μM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin, and 200 μM dNTP. After a hot start at 98 °C, 25 cycles (94 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min) of PCR were performed using 2.5 units of Thermus aquaticus (Taq) polymerase. The PCR products were subcloned into a pT7Blue-T-Vector (Novagen).

Isolation and DNA Sequencing of Porcine 1–6FucT cDNA

A porcine brain ST111 cDNA library (Clonetech) was screened by plaque hybridization utilizing the ϕX174-RD/CTP (3,000 Ci/mmol; Amer sham) of a labeled reverse transcription-PCR product, S1A12 (see Fig. 8). Five positive plaques were obtained from 4 × 10⁶ plaques in the first screening. The screening was repeated 3 times, and then the inserts of the five isolated clones were amplified using (GTTGTGCAGGTCAGGACGCGACGCGACGCG) and (GGTGTACCTCAGGACGAT GGAA) primers by means of PCR reaction, and the DNA sequence was directly determined with a DNA Sequencing Kit of Dye Terminator Cycle Sequencing Ready Reaction (Perkin-Elmer) using forward and reverse primers as sequencing primers. Based on the determined insert cDNA sequence, sequencing primers were synthesized, and the space between each sequencing primer was 200 nucleotides. Finally the full length of the insert cDNA sequence was determined.

| Steps          | Volume | Protein | Total activity | Specific activity | Purification | Yield % |
|----------------|--------|---------|----------------|------------------|--------------|---------|
| Triton X-100 extract | 75     | 3,680   | 2,220          | 0.6              | 1            | 100     |
| GnGn-bi-Asn-Sepharose 4B | 10     | 0.80    | 1,350          | 1,688            | 2,813        | 61      |
| GDP-CH2-6NH-Sepharose 4B | 0.2    | 0.01    | 262            | 262,200          | 437,000      | 12      |
Transient Expression of a1–6FucT in COS-1 Cells

The coding region of cDNA of a1–6FucT was amplified using forward (ggaattcGGAGTTGAAAGTCTGAAAATGCGG) and reversal (tccccc-ggggaCTTTCTCATCTGTCCGTC) primers by means of PCR reaction. The obtained PCR product was double digested by SmaI and EcoRI and subcloned into calf intestinal alkaline phosphatase-treated pSVK3, which was previously double digested by SmaI and EcoRI. pSVK3 was then transiently transfected into COS-1 cells by electroporation (Bio-Rad).

Northern Blotting Analyses of a1–6FucT mRNA in Rat Brain

Total RNA was prepared from rat cerebrum and cerebellum according to general methods. 20 μg of total RNA was chromatographed on a 0.8% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNA was transferred to a z-Probe membrane (Bio-Rad) by capillary action. After hybridization with 32P-labeled S-A12 fragment (see Fig. 9) at 42 °C, the membrane was washed at 55 °C in 2 x SSC (1 x SSC: 15 mM sodium citrate and 150 mM NaCl, pH 7.0) containing 0.1% SDS. The Kodak X-ray film was exposed for 3 days at -80 °C.

RESULTS

Purification and Column Chromatography of a1–6FucT

Steps 1 and 2—A porcine brain was chosen as a source of the enzyme for purification based on the fact that porcine and rat brains are the most abundant sources of the enzyme (Table I).

Step 3—The first chromatographic step of the purification involved fractionation on a GnGn-bi-Asn-Sepharose 4B column. GnGn-bi-Asn-Sepharose 4B chromatography resulted in a 61% yield and 2,800-fold purification (Table II). The majority of total protein loaded was eluted in the flow-through fraction of a column, as shown in Fig. 1. Although most glycosyltransferases and lectins were not retained by the column, porcine a1–6FucT is fully active in EDTA, as discussed below. From the beginning, enzyme activity began to be eluted gradually from the column, suggesting that the a1–6FucT exhibits heterogeneity. This is likely to be the reason why a 60% recovery of the total activity loaded was achieved when the column was eluted with KCl.
The purified enzyme was incubated under various conditions. The pH optimum was 7.0 (Fig. 4), and divalent cations had negligible effects on the activity. The enzyme is fully active in the presence of 5 mM EDTA. Ca^{2+} and Ni^{2+} strongly inhibited the activity of α1–6FucT.

### Structural analysis of enzymatic product

Relevant 1H NMR parameters of structural reporter groups of constituent monosaccharides for the enzymatic product are shown. Chemical shifts are given in ppm in D_{2}O (35 °C) containing 0.1% trifluoroacetic acid.

| Reporter group Residue | Chemical shift of enzymatic product |
|------------------------|-------------------------------------|
| H-1 GlcNAc-1           | 5.049                               |
| H-2 GlcNAc-1           | 5.049                               |
| H-2 GlcNAc-2           | ND*                                |
| H-2 GlcNAc-5           | 4.568                               |
| H-2 GlcNAc-5'          | 4.547                               |
| H-2 Man-3              | 4.765                               |
| H-2 Man-4              | 5.120                               |
| H-2 Man-4'             | 4.914                               |
| CH₃ α1–6Fuc            | 1.193                               |
| NAc GlcNAc-1           | 1.997                               |
| NAc GlcNAc-2           | 2.085                               |
| NAc GlcNAc-5–5’        | 2.051                               |

* ND, not detectable.

**Step 4**—After the buffer had been changed using an Amicon YM30, the final purification step was accomplished on a synthetic GDP-hexanolamine ligand column. Chromatography on an immobilized GDP-hexanolamine column resulted in an additional 155-fold purification with a 19.7% yield compared with the previous step. The elution profile from this column clearly showed that the majority of the contaminating protein was not bound to the column and that the enzyme was eluted from this column with GDP.

Starting with frozen porcine brain (100 g), α1–6FucT was purified 440,000-fold with a total yield of 12%, as summarized in Table II.

### Purity and Enzymatic Properties of α1–6FucT

In order to assess the level of purification of α1–6FucT, fractions eluted from a GDP-hexanolamine column were subjected to SDS-PAGE. A photograph of this gel, which was stained with Coomassie Brilliant Blue G-250 is shown in Fig. 2. The most purified sample in lanes 28 and 29 gave one major band corresponding to an apparent mass of 58 kDa (indicated by an arrow on the right).

The enzymatic profiles of α1–6FucT of porcine brain were obtained utilizing the purified protein. The purified enzyme was incubated by the enzyme assay method, and an aliquot was subjected to HPLC. A typical elution pattern is shown in Fig. 3. The substrate and product were eluted at 12.2 and 22.6 min, respectively. The product exhibits the same elution pattern as α1–6FucT of porcine liver (28). The enzymatic product was obtained utilizing the purified protein. The purified enzyme was incubated by the enzyme assay method, and an aliquot was subjected to HPLC. A typical elution pattern is shown in Fig. 3. The substrate and product were eluted at 12.2 and 22.6 min, respectively.

### Fig. 4. A, effect of pH on the α1–6FucT of porcine brain.

Incubation was performed at 37 °C for 30 min. The pH values were obtained with 250 mM acetate/NaOH (pH 4.0–5.2), Mes/NaOH (pH 5.6–7.0), and Tris/HCl (pH 7.1–8.5) buffer. B, effects of various divalent cations and EDTA on the α1–6FucT of porcine brain. The divalent cations, Mg^{2+} and Ca^{2+}, have negligible effects on the activity. The enzyme is fully active in the presence of 5 mM EDTA. Cu^{2+} and Ni^{2+} strongly inhibited the activity of α1–6FucT.

### Large Scale Purification and Column Chromatography of α1–6FucT

100 g of porcine brain was homogenized one time, and then extraction and purification of the enzyme were performed according to the method described in steps 1–3. These steps were repeated 20 times for approximately 2 kg of porcine brain, and the eluate from step 3 was collected and pooled in 20 mM potassium phosphate, pH 7.0, containing 1 mM KCl, 5 mM EDTA, and 0.1% Triton X-100. In this solution, α1–6FucT is fully stable. The pooled fractions were desalted against 20 mM potassium phosphate, pH 7.0, containing 50 mM KCl, and 5 mM EDTA, using a YM30 membrane, and step 3 was performed again for the total pooled fractions. The eluted fractions were collected and desalted against 20 mM potassium phosphate, pH 7.0, containing 50 mM KCl, and 5 mM EDTA, and then applied to a GDP-hexanolamine-Sepharose 4B column (16 × 5 cm;
Pharmacia HR16/10), which had been equilibrated with 20 mM potassium phosphate buffer containing 50 mM KCl, 5 mM EDTA, and 0.05% Triton X-100. After the column had been washed with the same buffers sufficiently, enzymes were eluted with 20 mM potassium phosphate buffer containing 50 mM KCl, 5 mM EDTA, 0.01% Triton X-100, and 5 mM GDP. The eluted fractions were collected and used for further experiments.

SDS-PAGE and Isoelectric Focusing of Purified α1–6FucT

The most purified fraction obtained on a GDP-hexanolamine affinity column migrates as one major component, 58 kDa, on SDS-PAGE under nonreducing conditions, and under reducing conditions the molecular mass of this component changes from 58 to 54 kDa (Fig. 5). The same component gave a diffuse band on isoelectric focusing at pl 7.0, and α1–6FucT activity was detected in the gel slices corresponding to this band (Fig. 6).

cDNA Cloning of α1–6FucT

The N-terminal sequence determined from a component of 54 kDa on SDS-PAGE was used to design sense strand PCR primers. The internal peptide sequence was determined from four lysyl endopeptidase-derived peptides (Fig. 7) and was used to design several antisense strand PCR primers. The five transcript-specific oligonucleotides obtained were used to amplify a cDNA that encodes the α1–6FucT (Fig. 7). The PCR product, S-A12, was then subcloned into pT7 Blue-Vector (Novagen), and the full-length sequence was determined (data not shown). From a λgt11 cDNA library of porcine brain (Clonetech), cDNA encoding α1–6FucT was also isolated, utilizing the PCR product as a probe. Five positive clones, C1, C2, C3, C4, and C5, were obtained as described in Fig. 8. Clones C1 and C2 appeared to contain the full-length α1–6FucT open reading frame, and the nucleotide sequence was determined from these two clones. The DNA sequence determined from the PCR product, S-A12, was completely identical to that of cDNA clone isolated from the λgt11 cDNA library. The translated sequence of α1–6FucT is shown in Fig. 9A. The 1,728-base pair open reading frame encodes a 575-amino acid polypeptide. The deduced amino acid composition was identical to that of the purified enzyme determined by amino acid analysis. Both the N-terminal and internal peptide amino acid sequences are included within the predicted sequence, as underlined in Fig. 9A.

Transient Expression of α1–6FucT in COS-1 Cells

To verify that the cloned cDNA encodes α1–6FucT transferase, the coding region of cDNA was subcloned into the mammalian expression vector, pSVK3, and then the vector was transfected into COS-1 cells. After 48 h, the cells were harvested, and α1–6FucT activity was measured in the homogenate. Preliminary experiments indicated that untransfected COS-1 cells show very little enzyme activity. COS-1 cells transfected with an expression vector containing α1–6FucT showed high activity, i.e., 2,360 pmol/h/mg of protein, compared with
the cells transfected with the control plasmid, mock pSVK3 (Table IV).

**Northern Blotting Analysis of Total RNA from Rat Brain**

Northern blotting analysis of total RNA from rat brain showed a band at 3.5 kilobases (Fig. 10). Brain showed one of the highest activities among various tissues, and the content of α1–6FucT mRNA of brain was also high.

**DISCUSSION**

We have reported the purification of N-acetylgalactosaminyltransferases III and V (31, 32) and employed donor and acceptor substrates for the sequential affinity chromatographies. In this paper we found that these chromatographies using donor and acceptor substrates as ligand were fairly effective for purification of α1–6FucT from porcine brain. Electrophoresis of the purified α1–6FucT gave a diffuse single band on the isoelectric focusing at pI 7.0 (Fig. 6). Based on the deduced amino acid composition from the cDNA sequence, the pI is 7.39. No putative N-glycosylation sites were found like several glycosyltransferases (33). On SDS-PAGE, a major band of 58 kDa appeared under nonreducing conditions. On reduction, however, the molecular mass changed to 54 kDa. Because of the limited material available for our study, the mechanism by which this change occurs is still unknown.

Weinstein et al. (34) reported that the amino terminus of glycosyltransferase was subjected to proteolytic cleavage due to an endogenous protease (32). According to the results of amino acid analysis, the cleavage site is very strict in the case of the N terminus of α1–6FucT as in the case of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase (35). It seems that the enzyme was cleaved by an endogenous protease during the extraction procedure.
complex oligosaccharide found in human transferrin (44). α-Fucose is presumed to play a role in N-glycan cycle and to determine the function of glycoproteins.

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upper panel

lower panel

FIG. 10. Northern blotting analyses of total RNA form rat brain. Total RNA from rat brain showed a faint band at 3.5 kilobases (upper panel). Ethidium bromide staining shows a comparable amount of RNA in each lane (lower panel).
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