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Purification and cDNA Cloning of GDP-L-Fuc:N-Acetyl-β-D-Glucosaminide:α1-6 Fucosyltransferase (α1-6 FucT) from Human Gastric Cancer MKN45 Cells[1]

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GDP-L-Fuc:N-acetyl-β-D-glucosaminide:α1-6 fucosyltransferase (α1-6 FucT), which catalyzes the transfer of fucose from GDP-Fuc to N-linked type complex glycopeptides, was purified from a culture supernatant of human gastric cancer cell line MKN45. The purification procedures included chromatographies on Q-Sepharose Fast Flow, synthetic GDP-hexanolamine-Sepharose, and GnGn-bi-Asn-Sepharose columns. SDS-PAGE of the purified enzyme gave a major band corresponding to an apparent molecular mass of 60 kDa. The enzyme was recovered in a 12% final yield with an approximately 4,600-fold increase in specific activity. The pH optimum was 7.5, and the enzyme was fully active in the presence of 5 mM EDTA and did not require divalent cations, Mg^{2+} and Ca^{2+}. Oligonucleotide primers designed from partial amino acid sequences were used to amplify and clone α1-6 FucT cDNA from a cDNA library of MKN45 cells. The cDNA encodes 575 amino acids in length, and contains the predicted N-terminal and internal amino acid sequences derived on lysyl endopeptidase digestion. The homology to porcine brain α1-6 FucT is 92.2% at the nucleotide level and 95.7% at the amino acid level. No putative N-glycosylation sites were found in the predicted amino acid sequence of the human MKN45 cell enzyme or that of porcine brain. Thus, the enzyme is distinct from other fucosyltransferases which catalyze α1-2, α1-3, and α1-4 fucose addition.

Key words: cDNA, fucosyltransferase, glycosyltransferase, N-glycan, purification.

For many glycopeptides, the carbohydrate structure is significantly altered during ontogenesis and oncogenesis (*1*), and is thought to play an important role in a wide variety of biological phenomena. In the case of the complex type sugar chains of *N*-glycans, the carbohydrate branching pattern is due to the cooperative participation of several glycosyltransferases, such as *N*-acetylglucosaminyltransferases (GnT-I-VI) and α1-6 fucosyltransferase (α1-6 FucT) (*2*). α1-6 FucT represents a pivotal enzyme in terms of the branching of *N*-glycans, and the enzyme was initially discovered and characterized in porcine liver (*3, 4*). α1-6 FucT attaches a fucose residue to the asparagine-linked GlcNAc (the inner-most GlcNAc) of *N*-glycans through an α1-6 linkage, and the resulting α1-6 fucose residue is preferentially recognized by lentil lectin (*5*). Differences in the pattern of binding of serum α-fetoprotein (AFP) with lentil lectin have been reported for hepatocellular carcinomas (HCC) and benign liver diseases (*6*). Analyses of the carbohydrate structure of AFP from HCC cell lines indicated that nearly all of the carbohydrate of AFP was α1-6 fucosylated (*7, 8*). AFP, produced by germ cell tumors, such as yolk sac tumors, also appears to be highly fucosylated (*9*). The activity of α1-6 FucT was higher in HCC liver tissue than in non-tumor tissue (*10*). These observations reinforce the biological significance of the role of α1-6 FucT in oncogenesis. α1-6 FucT from human fibroblasts of a cystic fibrosis (CF) patient was purified and characterized by Voynow *et al.* (*11*), but little is known about this enzyme and its gene regulation.

We recently reported the purification, characterization, and cloning of α1-6 FucT cDNA from porcine brain (*12*). This particular α1-6 FucT is a novel α1-6 FucT which is

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Abbreviations: α1-6 FucT, GDP-L-Fuc:*N*-acetyl-β-D-glucosaminide: α1-6 fucosyltransferase; PABA, 4-(2-pyridylamino)butyl amine; GDP-Fuc, guanosine diphospho-fucopyranoside; AFP, alpha-fetoprotein; ^{1}H NMR, proton nuclear magnetic resonance; GnGn-bi-Asn-PABA, GlcNAcβ1-2Manα1-6 (GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4 GlcNAc-Asn-PABA; GnGnF-bi-Asn-PABA, GlcNAcβ1-2Manα1-6 (GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (Fucα1-6) GlcNAc-Asn-PABA; MES, 2-(*N*-morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; bp, base pairs; CF, cystic fibrosis; HCC, hepatocellular carcinoma; CHAPS, 3((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate.

different from that from human fibroblasts (*11*), especially in terms of the pH optimum and molecular weight. The cDNA sequence of this enzyme was also determined for the first time.

In order to better understand the human α1-6 FucT produced by cancer cells, we have purified α1-6 FucT from the cultured supernatant of a human gastric cancer cell line, MKN45, using purification procedures different from those used in the case of porcine liver, and characterized the enzyme. In addition, cDNA for human α1-6 FucT was also isolated from a cDNA library of MKN45 cells, revealing that the cDNA sequence is highly conserved between human MKN45 cells and porcine brain.

EXPERIMENTAL PROCEDURES

Materials—2-Amino pyridine was obtained from Wako Pure Chemicals, Osaka. Bovine γ-globulin and pronase (*Streptomyces griseus*) were purchased from Sigma and Seikagaku Kogyo, Tokyo, respectively. Sialidase (*Arthrobacter ureafaciens*) was a generous gift from Dr. Y. Ohta, Marukin Shoyu, Kyoto. β-Galactosidase (*Aspergillus* sp.) was obtained from Toyobo. The TSK-gel ODS-80TM and Amide-80 columns were purchased from Tosoh, Tokyo. Q-Sepharose Fast Flow was obtained from Pharmacia LKB Biotechnology. DNA restriction enzymes were obtained from Toyobo. The vectors used in this work were as follows: pBluescript SK− phagemid vectors from Stratagene, pT7BlueT-vectors from Novagen, and pSVK3 expression vectors from Pharmacia LKB Biotechnology.

α1-6 FucT Assay—Enzyme activity was determined as described previously, using a fluorescence-labeled sugar chain, GnGn-Asn-PABA, as an acceptor substrate (*13*). Protein was determined using a Bio-Rad protein assay kit with bovine serum albumin as a standard.

The optimum pH of α1-6 FucT of MKN45 was estimated as follows. After incubation of the enzyme at 37°C for 1 h at several pHs, α1-6 FucT activity was assayed. The pHs of the solutions were obtained with 200 mM MES-NaOH (pH 5.5-7.5) and 50 mM Tris-HCl (pH 7.0-9.0) buffer.

The optimum temperature of α1-6 FucT of MKN45 was estimated by incubating the enzyme with 200 mM MES-NaOH (pH 7.5) at several temperatures (20-55°C) for 1 h.

The pH stability of α1-6 FucT of MKN45 was estimated as follows. The purified enzyme was incubated at several pHs at 4°C for 5 h. After incubation of the enzyme, the remaining activity was measured. The pHs were obtained with 20 mM acetate-NaOH (pH 3.5-5.5), 20 mM MES-NaOH (pH 5.0-7.5), 20 mM Tris-HCl (pH 7.5-9.0), and 20 mM carbonate-bicarbonate (pH 9.0-11.0) buffer.

Purification and Column Chromatography of α1-6 FucT—Preparation of culture supernatants: The human stomach cancer cell line, MKN45, obtained from the Japanese Cancer Research Resources Bank, No. JCRB-0254, was cultured in a roller bottle. Cells (1.5×10^7) were inoculated into a roller bottle with a usable surface area of 225 cm^2 and then grown to confluence in 400 ml of complete medium (Ham's F12 medium : RPMI 1640 medium = 1 : 1, containing 5% calf serum and 50 μg/ml kanamycin). After 7 days culture, the medium was changed to a serum-free medium containing 10^{-8} M sodium selenite. The medium was changed every 4 days and the supernatants were pooled at 4°C. One hundred liters of the culture supernatant was concentrated to 2 liters, and then exchanged by ultrafiltration with 20 mM Tris-HCl, pH 7.5, containing 0.1% CHAPS, 5 mM 2-mercaptoethanol, and 0.1 M NaCl.

Removal of Nucleic Acids with Streptomycin Sulfate—Nucleic acids, which interfere with chromatography, were removed by precipitation with streptomycin sulfate. Streptomycin sulfate (1.0%) was gradually added to the above mixture to give a final concentration of 0.15%. The mixture was then stirred at 4°C for 30 min, followed by centrifugation at 10,000 × *g* for 30 min. The supernatant was dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 0.1% CHAPS and 5 mM 2-mercaptoethanol using a YM30 membrane (Amicon).

Q-Sepharose fast flow chromatography: The supernatant was applied to a column of Q-Sepharose Fast Flow (2.5 cm × 18 cm) which had been equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 0.1% CHAPS and 5 mM 2-mercaptoethanol, which was then washed with 1 liter of equilibration buffer. Elution was carried out using a buffer containing 0.1 M NaCl.

GDP-hexanolamine-Sepharose 4B chromatography: The enzymatically active fractions obtained with the Q-Sepharose Fast Flow column were collected, desalted by dialysis against 20 mM Tris-HCl buffer, pH 7.5, containing 0.7% CHAPS, and 5 mM 2-mercaptoethanol using a YM30 membrane, and then applied to a GDP-hexanolamine-Sepharose 4B column (1 cm × 7.6 cm) which had been equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 0.7% CHAPS and 5 mM 2-mercaptoethanol. After a sufficient wash with the same buffer, the enzyme was eluted with a linear concentration gradient of 0 to 0.5 M NaCl in the buffer.

GnGn-bi-Asn-Sepharose 4B chromatography: The enzymatically active fractions obtained with the GDP-hexanolamine-Sepharose 4B column were collected, desalted by dialysis against 20 mM Tris-HCl buffer, pH 7.5, containing 0.7% CHAPS and 5 mM 2-mercaptoethanol using a YM30 membrane, and then applied to a GnGn-bi-Asn-Sepharose 4B column (1 cm × 7.6 cm) which had been equilibrated with the buffer used for desalting. After a sufficient wash with the same buffer, the column-bound enzyme was eluted with a linear concentration gradient of 0 to 0.5 M NaCl in the buffer.

SDS-Polyacryl Amide Gel Electrophoresis (PAGE)—SDS-PAGE was performed by the method described by Laemmli (*14*) in the presence or absence of 2-mercaptoethanol. The gel was stained with Coomassie Brilliant Blue R-250. α-Lactalbumin (M_r 14,400), soybean trypsin inhibitor (M_r 20,100), carbonic anhydrase (M_r 30,000), ovalbumin (M_r 43,000), bovine serum albumin (M_r 67,000), and phosphorylase *b* (M_r 94,000) were used as molecular weight standards.

Determination of Partial Amino Acid Sequences of α1-6 FucT—Ten micrograms of purified protein was subjected to SDS-PAGE, followed by electroblotting onto a PVDF membrane (Millipore). To obtain N-terminal amino acid sequences, the material corresponding to the 60 kDa band was excised from a PVDF membrane and then sequenced with an Applied Biosystem 473A Protein Sequencer. To obtain internal amino acid sequences, 10 μg of purified protein and 50 ng of lysyl endopeptidase were mixed, followed by SDS-PAGE. After 30 min electrophoresis, the current was interrupted for 2 h at room temperature for

digestion of the protein, and then reapplied to separate the peptides. The resultant peptides were transferred to a PVDF membrane and detected with Coomassie Brilliant Blue G-250. Two peptides were obtained and excised from the PVDF membrane, and then sequenced as above.

Construction of cDNA—Total RNA was isolated from cultured MKN45 cells. Poly(A)$^+$RNA was further purified utilizing a MESSAGE MARKER™ Reagent Assembly (GIBCO BRL). The initial cDNA strand for RT-PCR was synthesized with a cDNA Synthesis Kit (GIBCO BRL) using a random primer according to the manufacturer's protocol.

Polymerase Chain Reaction (PCR)—Four oligonucleotides were synthesized for use as primers in PCR (Fig. 3B). PCR was carried out in 50 μl of a solution which comprised an amount of initial strand cDNA corresponding to 2 μg Poly(A)$^+$RNA, 50 pmol of a pair of degenerate oligonucleotides, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001% gelatin, and 200 μM dNTP. After a hot start at 98°C, 36 cycles (one cycle: 94°C for 30 s, 46°C for 30 s, and 72°C for 90 s) of PCR were performed using 2.5 units of *Thermus aquaticus* (Taq) polymerase. The PCR products were subcloned into a pT7BlueT-vector.

Construction of a MKN45 λZAP cDNA Library—A MKN45 λZAPII cDNA library was constructed using a ZAP-cDNA Synthesis Kit (Stratagene) according to the manufacturer's protocol.

Isolation and DNA Sequencing of Human α1-6 FucT cDNA—The MKN45 λZAPII cDNA library was screened by plaque hybridization utilizing the [γ-^{32}P]dCTP (3,000 Ci/mmol, Amersham) labeled RT-PCR product, L2S-L1A (Fig. 3, A and B). On the third screening, eight positive plaques were obtained from among 2×10^6 plaques. The inserts of the positive clones were digested with several restriction enzymes, and each separated fragment was subcloned into pBluescript SK. The DNA sequence was determined with a DNA Sequencing Kit for Dye terminator Cycle Sequencing (Perkin-Elmer) using T3 and T7 primers as sequencing primers.

RESULTS

Purification and Column Chromatography of α1-6 FucT—Starting with the culture supernatant of MKN45, 3,860 mg, α1-6 FucT was purified 4,634-fold in a total yield of 12%, as summarized in Table I. Streptomycin sulfate was used to remove nucleic acids that interfered with Q-Sepharose Fast Flow chromatography. The streptomycin sulfate treatment resulted in a 1.3-fold purification and a 92% yield.

The first chromatography step for the purification involved fractionation by anion exchange chromatography on Q-Sepharose Fast Flow. The samples were loaded and enzymatically active fractions were obtained by elution with a buffer containing 0.1 M NaCl (Fig. 1A). Q-Sepharose Fast Flow chromatography resulted in a 3.3-fold purification and a 68.5% yield. The enzymatically active fractions were collected and desalted against 20 mM Tris-HCl buffer, pH 7.5, containing 0.7% CHAPS and 5 mM 2-mercaptoethanol using a YM30 membrane. Although α1-6 FucT in the culture supernatant was not thought to be a membrane-bound form of the protein, CHAPS, which is an ampholytic surfactant, was required to stabilize the small amount of enzyme obtained.

The second purification step was performed on a synthetic GDP-hexanolamine ligand column. GDP-hexanolamine was synthesized as described previously with the following modification (*15*). GMP was activated with morphoridate and coupled to 6-aminohexylphosphate. The end product was completely purified on a TSK gel ODS TM80 column (4.6 mm×150 mm). The structure of GDP-hexanolamine was confirmed by ^{1}H NMR (data not shown). The sample was loaded and the enzyme activity was eluted with a gradient of 0.2-0.4 M NaCl (Fig. 1B). Chromatography on an immobilized GDP column resulted in an additional 16.6-fold purification and a 69.8% yield, compared to the Q-Sepharose column step.

For the final purification step GnGn-bi-Asn-Sepharose 4B chromatography was used. The enzymatically active fractions comprised the 0.25-0.5 M NaCl eluted fraction, as shown in Fig. 1C. Chromatography on GnGn-bi-Asn-Sepharose 4B resulted in an additional 65.5-fold purification and a 27.3% yield, compared to GDP-hexanolamine-Sepharose column step. Overall, 100 μg of α1-6 FucT was obtained as the final product.

Purity and Enzymatic Properties of α1-6 FucT—To assess the level of purification of α1-6 FucT, fractions eluted from the GnGn-bi-Asn-Sepharose 4B column were subjected to SDS-PAGE and then stained with Coomassie Brilliant Blue R-250 (Fig. 2). The purified α1-6 FucT from the GnGn-bi-Asn-Sepharose 4B column migrates as one major component on SDS-PAGE, corresponding to 60 kDa under both reducing and non-reducing conditions. Conversion of the major band material to a 54 kDa component, as observed in the reduced α1-6 FucT from porcine brain, was not detected in the case of α1-6 FucT from MKN45 cells (data not shown). The purified enzyme was characterized under a variety of conditions. The optimum pH was 7.5 and the optimum temperature was 30-37°C (Table II). The enzyme was stable in the pH range of 4.0-10.0 (Table II). The enzyme was assayed after 1 h incubation with various divalent cations at 10 mM or EDTA at 10 mM. The presence of divalent cations such as Mg^{2+} and Ca^{2+} had a negligible effect on the activity, and the enzyme was fully active even in the presence of 5 mM EDTA at pH 7.5 (data not shown). Some divalent cations such as Co^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+}, suppressed the enzyme activity (data not shown).

cDNA Cloning of α1-6 FucT—The internal peptide sequence was obtained from two lysyl endopeptidase-derived peptides (Fig. 3A), and used to design sense and anti-sense PCR primers (Fig. 3B). Agarose gel electrophoresis of the PCR products showed that only the combination of primers L2S (sense) and L1A (antisense) produced a

TABLE I. **Purification of α1-6 FucT from MKN45 cells.**

Step	Protein (mg)	Total activity (nmol/h)	Specific activity (nmol/h/mg)	Purification fold (factor)	Yield (%)
Culture medium	3,860	24,800	6.4	1	100
Streptomycin sulfate	2,730	22,800	8.4	1.3	92
Q-Sepharose	570	15,600	27.0	4.3	63
GDP-hexanolamine-Sepharose	24	10,900	450	71.0	44
GnGn-bi-Asn-Sepharose	0.1	2,980	29,800	4,630	12

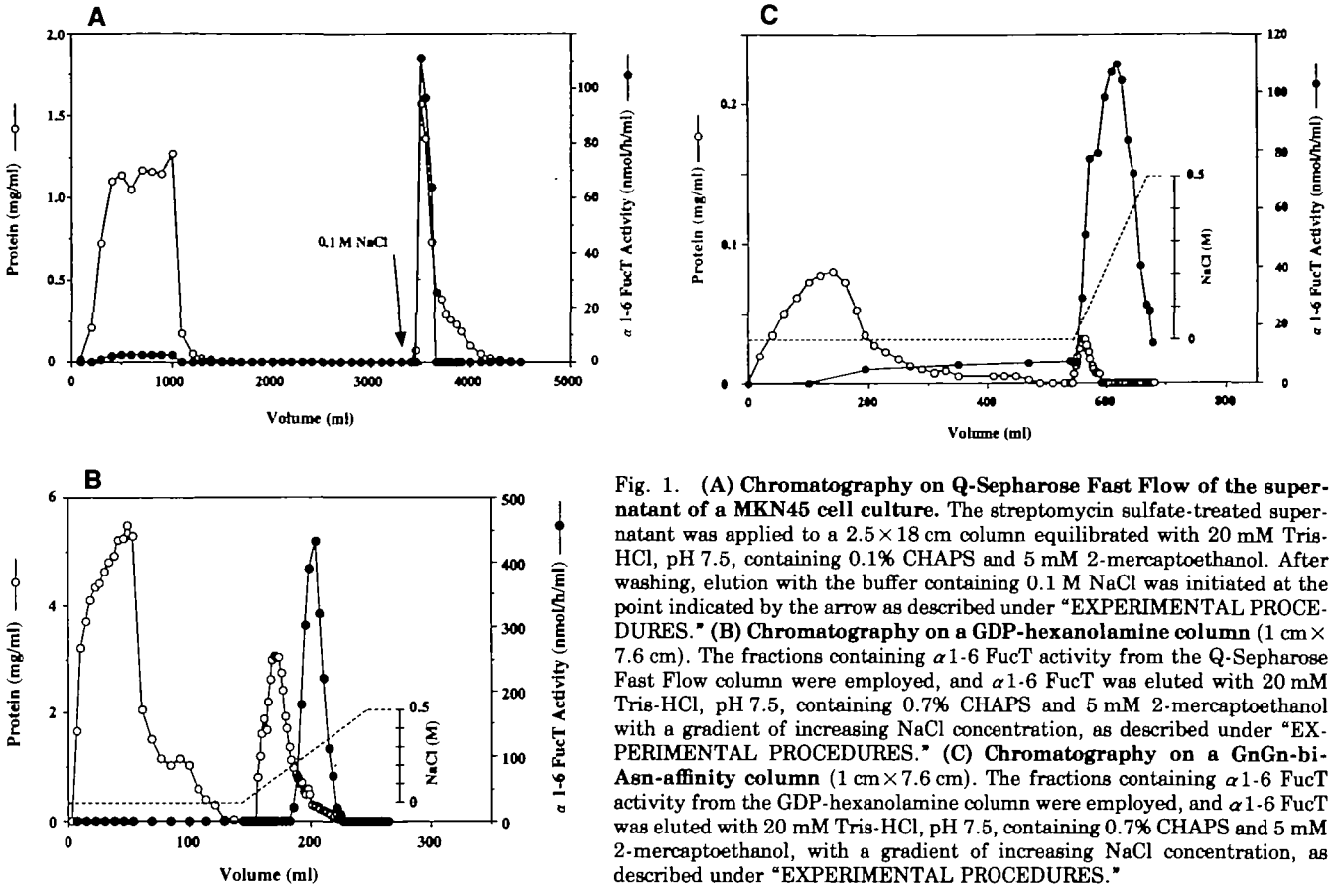

Fig. 1. **(A) Chromatography on Q-Sepharose Fast Flow of the supernatant of a MKN45 cell culture.** The streptomycin sulfate-treated supernatant was applied to a 2.5×18 cm column equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.1% CHAPS and 5 mM 2-mercaptoethanol. After washing, elution with the buffer containing 0.1 M NaCl was initiated at the point indicated by the arrow as described under "EXPERIMENTAL PROCEDURES." **(B) Chromatography on a GDP-hexanolamine column** (1 cm× 7.6 cm). The fractions containing α1-6 FucT activity from the Q-Sepharose Fast Flow column were employed, and α1-6 FucT was eluted with 20 mM Tris-HCl, pH 7.5, containing 0.7% CHAPS and 5 mM 2-mercaptoethanol with a gradient of increasing NaCl concentration, as described under "EXPERIMENTAL PROCEDURES." **(C) Chromatography on a GnGn-bi-Asn-affinity column** (1 cm×7.6 cm). The fractions containing α1-6 FucT activity from the GDP-hexanolamine column were employed, and α1-6 FucT was eluted with 20 mM Tris-HCl, pH 7.5, containing 0.7% CHAPS and 5 mM 2-mercaptoethanol, with a gradient of increasing NaCl concentration, as described under "EXPERIMENTAL PROCEDURES."

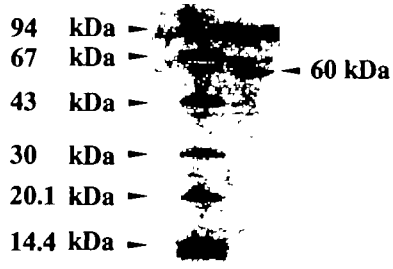

Fig. 2. **Electrophoresis of the purified α1-6 FucT on a 10-15% SDS-polyacrylamide gel under reducing conditions.** The gel was stained with Coomassie Brilliant Blue R-250. α-Lactalbumin (M_r 14,400), soybean trypsin inhibitor (M_r 20,100), carbonic anhydrase (M_r 30,000), ovalbumin (M_r 43,000), bovine serum albumin (M_r 67,000), and phosphorylase *b* (M_r 94,000) were used as molecular weight standards (left lane). The apparent molecular mass of α1-6 FucT is 60 kDa (right lane).

DNA fragment of 200 bp (data not shown). The PCR product, L2S-L1A, was then subcloned into a pT7Blue-vector and the full length sequence was determined (data not shown). From a λZAPII cDNA library of MKN45, cDNA encoding α1-6 FucT was also isolated using the PCR product, L2S-L1A, as a probe. Eight positive clones (c1-c8) were obtained. Clones c1-c7 appeared to contain the full open reading frame of α1-6 FucT and the nucleotide sequence was determined using clone c1. The DNA sequence obtained from the PCR product, L2S-L1A, was

TABLE II. **Characteristics of the purified human α1-6 FucT.**

	MKN45	Porcine brain[a]	Fibroblasts (CF)[b]
Molecular mass (kDa)	60	58	34 and 39
Optimum pH[c]	7.5	7.0	5.5
Optimum temperature (°C)[d]	30-37	30-37	–
pH stability[e]	4-10	4-10	–

[a]The values are taken from the data of Uozumi *et al.* (*12*). [b]The values are taken from the data of Voynow *et al.* (*11*). [c]Optimum pH, [d]optimum temperature, and [e]pH stability of α1-6 FucT of MKN45 were estimated as described under "EXPERIMENTAL PROCEDURES."

completely identical with those of the cDNA clones isolated from the cDNA library. The nucleotide and predicted amino acid sequences of human α1-6 FucT are shown in Fig. 4. The 1,728 bp open reading frame encodes a 575 amino acid polypeptide. The deduced amino acid composition was comparable with that of the purified enzyme determined by amino acid analysis. Both the N-terminal and internal peptide amino acid sequences are included in the predicted sequence, as underlined in Fig. 4. There were no potential *N*-glycan sites in the sequence. The homology to porcine brain α1-6 FucT is 92.2% at the nucleotide level and 95.7% at the amino acid level. No sequence homology was found with other fucosyltransferases.

Transient Expression of α1-6 FucT in COS-1 Cells—To verify that the cloned cDNA encodes α1-6 FucT, the coding region of the cDNA was subcloned into the mammalian

A

N-terminal RIPEGPIDQGPAIGRVRVL

L1 peptide TKIPNYEFISDNSIS

L1S

L1A

L2 peptide KLGFKHPVIGVHVRRTDKVGTEAAF

L2S

L2A

B

L1S	5'- ACHAARTAYCCHAAYTAYGA -3'
L1A	5'- TCRTARTTDGGRTAYTTDGT -3'
L2S	5'- TTYAARCAYCCHGTBATYGG -3'
L2A	5'- CCRATVACDGGRTGYTTRAA -3'

Fig. 3. **Partial amino acid sequences of the lysyl endopeptidase-derived peptides (A) of α1-6 FucT and oligonucleotide primers (B) based on the peptide sequences.** A: The underlined sections of the peptide sequences were used to design oligonucleotide primers. B: Oligonucoleotides L1S and L1A, and L2S and L2A were based on L1 and L2, respectively. R: A or G, Y: C or T, B: C or G or T, D: A or G or T, H: A or C or T, V: A or C or G.

expression vector, pSVK3. After 48 h transfection of the α1-6 FucT expression vector into COS-1 cells, the cells were harvested and α1-6 FucT activity was measured in the cell homogenate. Naive COS-1 cells exhibit very little α1-6 FucT activity (13 pmol/h/mg). COS-1 cells transfected with 50 μg of the α1-6 FucT cDNA showed an activity level of 2,130 pmol/h/mg of protein, compared with an activity level of 15 pmol/h/mg of protein for cells which had been transfected with 50 μg of the control plasmid, mock pSVK3.

DISCUSSION

α1-6 FucT catalyzes the transfer of fucose to asparagine-linked GlcNAc at an early stage of complex type *N*-glycan biosynthesis. An increase in the α1-6 fucosylation of *N*-glycans is known to be highly correlated with hepatocarcinogenesis and thus suggests the pathophysiological significance of this structure (*6-10*). To investigate the regulation of α1-6 fucosylation by α1-6 FucT in human diseases, α1-6 FucT activity was screened in a number of cell lines, in order to identify a source for this enzyme. α1-6 FucT activity was very high in several human cell lines, including myeloma, pancreatic cancer and lung cancer cell lines (data not shown). Among them, a human gastric cancer cell lines MKN45, was found which secretes α1-6

Fig. 4. **Nucleotide and amino acid sequences of human α1-6 FucT.** Single-letter notations are used for amino acids. The sequences of the lysyl endopeptidase-derived peptides are underlined with single solid lines. The proposed transmembrane region is double underlined.

FucT activity into the culture medium, and therefore was selected as a source for the production of the enzyme.

The cultured supernatant of human gastric cancer cell line MKN45 was used as a source for the enzyme purification for the following reasons. Firstly, a high level of α1-6 FucT activity was detected in the supernatant of MKN45 cultures. Secondly, the starting material was not highly contaminated by protein because fetal bovine serum is not essential for the growth of these cells. Thirdly, no detergent is required for solubilization of the enzyme because α1-6 FucT is secreted into the medium in a soluble form.

In the present study, three types of columns, *i.e.* Q-Sepharose, GDP-hexanolamine-Sepharose 4B and GnGn-bi-Asn-Sepharose 4B affinity columns, were used to purify α1-6 FucT from the serum-free culture medium. The GnGn-bi-Asn-Sepharose 4B and GDP-hexanolamine-Sepharose 4B column chromatographies resulted in a high level of purification.

SDS-PAGE gave a major 60 kDa band under both reducing and non-reducing conditions. Uozumi *et al.* recently reported the purification and cDNA cloning of α1-6 FucT from porcine brain (*12*), and showed that a major 58 kDa band was converted to a 54 kDa band under reducing conditions. Such a conversion was, however, not observed in the present enzyme under reducing conditions, and further conformational characterization or comparison of these purified enzymes will be required to explain this difference.

The amino terminus of glycosyltransferase is known to be subject to proteolytic cleavage by endogenous protease (*16-18*). In the case of the N-terminal amino acid of α1-6 FucT from MKN45 cells, this cleavage site was identified. Compared with the enzyme of porcine brain, the cleavage site of the MKN45 enzyme is 34 residues upstream of that of the porcine brain enzyme (*12*), which is consistent with the difference in the molecular sizes of the enzymes.

The pH optimum of the MKN45 enzyme was 7.5, and its temperature optimum was 30-37°C (Table II). Divalent cations, such as Mg^{2+} and Ca^{2+}, were found to have a negligible effect on the activity, and the enzyme was fully active in the presence of 5 mM EDTA at pH 7.5. The presence of other cations, such as Co^{2+}, Ni^{2+}, Cu^{2+}, and Cd^{2+}, suppressed the enzyme activity. The mechanisms by which some cations suppress the enzyme activity are not clear at present. One possibility is that thiol group(s) of the enzyme would be oxidized in the presence of the above cations (*19*), which would result in inactivation of the enzyme. These characteristics of the purified MKN45 enzyme are very similar to those of the porcine brain enzyme (*12*), but different from those of the purified α1-6 FucT of human skin fibroblasts reported previously (*11*) in terms of pH optimum (pH 5.5) and molecular size (34 and 39 kDa). These results suggest that α1-6 FucTs may be classified into several groups, and the MKN45 and porcine brain enzymes may belong to the same group because they are very similar in terms of enzyme characteristics.

Eight independent cDNA clones for human α1-6 FucT were isolated using the PCR product derived from partial amino acid sequences of the purified enzyme of MKN 45 cells (Fig. 3). A restriction map of all the clones showed the same pattern with restriction enzymes such as *Eco*RI, *Pst*I, *Sac*I, and *Hin*dIII (data not shown). Partial DNA sequencing of each clone and the sizes of the clones showed that clones c1-c7 appeared to contain the full length α1-6 FucT open reading frame (data not shown), and the nucleotide sequence of the longest clone, c1, was determined for both the 5′- and 3′-sides.

The enzyme has a domain structure similar to those of other glycosyltransferases; α1-6 FucT also has a short amino terminal cytoplasmic tail, a 21 amino acid transmembrane sequence, a "neck" region, and a long C-terminal catalytic portion, which is in the Golgi lumen (*20*) (Fig. 4). The enzyme has a proline-rich domain, with 10 proline residues between residues 261 to 340 (Fig. 4). As judged from a hydropathy plot, a hydrophobic region which preceeds the amino terminus by 9 amino acids is likely to represent the transmembrane region of this enzyme (data not shown). No sequence homology was found between MKN45 α1-6 FucT and other fucosyltransferases (*21*), but α1-6 FucT of the human gastric cancer cell line exhibited very high homology to that of porcine brain (92.2% at the nucleotide level and 95.7% at the amino acid level), and the total number of amino acids was identical (575 amino acids). At present it is not clear whether MKN45 α1-6 FucT is a cancer-specific enzyme or not.

In the case of the α1-3 FucT family, the conservative positions of cysteine residues in the sequence were suggested to be important for the enzyme function (*22*). In the case of α1-6 FucT, the positions of cysteine residues are also completely conserved in both sequences, and 2-mercaptoethanol was also used as an antioxidant during purification, since α1-6 FucT is sensitive to oxidation (data not shown). These findings suggest that the cysteine residues in α1-6 FucT may play important roles in the enzyme function. Taken together, the high similarity between the MKN45 enzyme and that from porcine brain is compatible with the characteristics of these enzymes (Table II). Moreover, another characteristic of this glycosyltransferase is that no potential *N*-linked glycosylation site is present in the deduced amino acid sequence, suggesting that the active enzyme could be overexpressed using bacterial systems.

Herein we have described the purification, characterization, and cDNA cloning of human α1-6 FucT. The cDNA will be used in further studies on the pathophysiological regulation of human α1-6 FucT gene expression.

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