CMP-NeuAc:Galβ1→4GlcNAc α2→6sialyltransferase catalyzes NeuAc transfer to glycolipids

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Abstract Using mammalian gene-overexpression system, in vitro catalytic activities of CMP-NeuAc:Galβ1→4GlcNAc α2→6sialyltransferase on glycosphingolipid acceptors were analyzed. We transfected the mammalian expression vector containing the cDNA that was cloned from Daudi cells into COS-1 cells, and selected monoclonal transfectants in the presence of G418. Although the transfectedα2→6sialyltransferase can catalyze NeuAc transfer onto glycoprotein acceptors more than glycolipids based on kinetic analyses, the substantial synthesis of IV^{6}NeuAc-nLcOse$_4$Cer was observed and the activities were 7- to 9-times higher in the transfected cells than in the mock transfectants. In addition, the transfected COS-1 cells with α2→6sialyltransferase cDNA were revealed to contain a higher amount of ganglioside that has the terminal NeuAcα2→6Gal sequence in the in situ situation than the mock transfectants. These results using transfectants, together with those using the purified enzyme protein, suggest that the α2→6sialyltransferase enzyme from Daudi cells can also catalyze NeuAc transfer in α2→6 linkage onto glycosphingolipid acceptors.—**Nakamura, M., A. Tsunoda, K. Yanagisawa, Y. Furukawa, J. Kikuchi, A. Iwase, T. Sakai, G. Larson, and M. Saito.** CMP-NeuAc:Galβ1→4GlcNAc α2→6sialyltransferase catalyzes NeuAc transfer to glycolipids. *J. Lipid Res.* 1997. **38:** 1795–1806.

Supplementary key words sialyltransferase • glycosphingolipid • substrate specificity • cDNA transfection • stable expression • gene overexpression

Glycosphingolipids and glycoproteins have been implicated and demonstrated to play important roles in cell growth, cell differentiation, cell adhesion, morphogenesis, and binding with toxins, bacteria, and viruses (3–9). The biosynthesis of the carbohydrate sequences on a ceramide moiety is accomplished through the action of sequential glycosyltransferases that transfer sugar residues from sugar nucleotides to an oligosaccharide chain attached to ceramide (10). After transfer from dolichol phosphate-oligosaccharides to an Asn residue of protein moiety and hydrolase trimming, elongation of the oligosaccharide chain is also conducted through the action of the sequential glycosyltransferases for *N*-glycan.

In leukocytes and leukemia cell lines, sugar sequences are expressed in a lineage-specific manner and are thought to play a critical role in cell differentiation (11–14) as well as in the production of ligands for cell adhesion molecules in some cell lineages (8). In addition, structures and functions of cell surface-differentiation antigens have been intensively studied, and some of them, including CD15, CDw17, CD57, CDw60, CDw65, and CD77, are also classified as cell surface carbohydrate determinants (15). Among them, the epitopes of leukocyte differentiation antigen clusters CDw75 and CD76 have been proved to be generated through the action of CMP-NeuAc:Galβ1→4GlcNAc α2→6sialyltransferase [EC 2.4.99.1] (α2→6ST), (16–19). A recent study has shown that CD76 is an α2→6 sialylated lacto-series motif found on glycolipids (20).

Abbreviations: α2→6ST, CMP-NeuAc:Galβ1→4GlcNAc α2→6sialyltransferase [EC 2.4.99.1]; α1AGP, α1-acid glycoprotein; ASα1AGP, asialo-α1-acid glycoprotein; nLc$_4$-HSA, nLcOse$_4$-conjugated human serum albumin; PA, pyridylaminated; FITC, fluorescein isothiocyanate; 2-ME, 2-mercaptoethanol; NDV, Newcastle disease virus. Glycosphingolipids are designated according to the recommendation of the Nomenclature Committee of the IUPAC (1) and gangliosides are designated as described (2): nLc$_4$Cer, nLcOse$_4$Cer; nLc$_6$Cer, nLcOse$_6$Cer; Lc$_4$Cer, LcOse$_4$Cer; Lc$_3$Cer, LcOse$_3$Cer.

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The α2→6ST was purified and cloned from rat liver (21, 22). This sialyltransferase has been purified using glycoprotein acceptor and its catalytic reaction on glycoprotein was demonstrated. However, it has remained uncertain whether the same α2→6ST may act on both glycosphingolipids and glycoproteins containing the same sugar terminal sequence, Galβ1→4GlcNac. In general, some glycosyltransferases act only on glycoproteins such as UDP-GlcNAc:α1→3mannosyl glycoprotein β1→2-*N*-acetylglucosaminyltransferase I (23), while other glycosyltransferases act only on glycosphingolipids such as UDP-GalNAc:GM3/GD3 β1→4-*N*-acetylgalactosaminyltransferase (GM2/GD2 synthase) (24). In some glycosylation steps, however, the same glycosyltransferases may catalyze reactions on both glycoproteins and glycosphingolipids such as UDP-Gal: GlcNAcβ1→3Gal β1→4galactosyltransferase (25, 26). It is of interest to investigate in which group of the above the α2→6ST is classified, and whether the sialyltransferase catalyzes NeuAc transfer onto glycolipid acceptors. Using mammalian gene-overexpression system, we present here experimental evidence that the α2→6sialyltransferase could also catalyze NeuAc transfer onto glycosphingolipid acceptors.

EXPERIMENTAL PROCEDURES

Materials

Rat liver α2→6ST was purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and further purified by CDP-ethanolamine-conjugated agarose chromatography. CMP-[sialic-4,5,6,7,8,9-^{14}C]NeuAc (300.9 mCi/mmol) and unlabeled CMP-NeuAc were obtained from New England Nuclear (Boston, MA) and from Sigma (St. Louis, MO), respectively. Asialo-α1-acid glycoprotein (ASα1AGP) and asialo-transferrin were prepared from α1-acid glycoprotein (α1AGP) and transferrin (both from Sigma) by mild acid hydrolysis (27), respectively. Possible acceptor oligosaccharide numbers of an ASα1AGP molecule were estimated as NeuAc free oligosaccharides comparing the NeuAc concentration of α1AGP by resorcinol/HCl method before and after mild acid hydrolysis. Synthetic glycoprotein nLcOse$_4$-human serum albumin (nLc$_4$-HSA) was purchased from BioCarb (Lund, Sweden). nLcOse$_4$Cer (nLc$_4$Cer) and nLcOse$_6$Cer (nLc$_6$Cer) were prepared by desialylation of IV^{3}NeuGc–nLc$_4$Cer and VI^{3}NeuGc–nLc$_6$Cer that were purified from bovine erythrocytes (28). LcOse$_4$Cer was purified from human meconium as described (29). Pyridylaminated oligosaccharides, nLc$_4$-PA, Lc$_4$-PA, lactose-PA, Galβ1→3GlcNAc-PA, and Galβ1→4GlcNAc-PA, were prepared as described (30). mAb NS-24, that recognizes a terminal NeuAcα2→3Galβ1→4GlcNAc sugar sequence (31), was a generous gift from Dr. Yasuo Suzuki (Shizuoka University, Shizuoka, Japan). mAb 1B9, that recognizes a terminal NeuAcα2→6Gal sequence (32), was generously donated by Dr. Sen-itiroh Hakomori (Biomembrane Institute, Seattle, WA). Newcastle disease virus (NDV) neuraminidase and *Arthrobacter ureafaciens* (*A. ureafaciens*) neuraminidase were purchased from Sigma. FITC-conjugated goat F(ab′)$_2$ anti-mouse IgM antibody was from TAGO Inc. (Burlingame, CA), and rabbit anti-mouse IgM antibody and mouse anti-human IgG mAb (class IgM) were from Sigma. Guanidinium thiocyanate was purchased from Fluka (Buchs, Switzerland) and CsCl was from Nakarai Tesque (Kyoto, Japan). Biodyne B nylon membrane was from Nippon Genetics (Tokyo, Japan). All other reagents were of the highest grade commercially available.

Cells and cell cultures

Human Burkitt lymphoma cell line Daudi, human myeloma cell line U266, and African green monkey's kidney cell line COS-1 cells were cultured in RPMI-1640 medium supplemented with 20% fetal calf serum, RPMI-1640 medium supplemented with 10% fetal calf serum, and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, respectively. The cells were harvested, centrifuged, washed twice with phosphate-buffered saline, and stored at −80°C until use.

PCR-cloning of human α2→6ST cDNA from Daudi

Human α2→6ST cDNA was cloned from Daudi cDNA, that was reverse-transcribed from poly(A)-tailed RNA, by PCR using synthetic oligonucleotide primers complementary to sequences 5′ and 3′ of the coding region. The primers were as follows:

sense: AAT-AAA-CCT-TCC-CTC-CCA-TGG-A

antisense: TTA-GCA-GTG-AAT-GGT-CCG-GAA

Reactions were done using AmpliTaq DNA polymerase (Perkin-Elmer). Thirty cycles of amplification were carried out along the following scheme: 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. At the final cycle, the reactions were completed at 72°C for 7 min. A fraction of the product was examined on a 0.8% agarose gel, and a part of the remaining was cloned to pCRII vector using T–A overhang (Invitrogen, San Diego, CA). The *Hin*dIII/*Apa*I fragment of sense-oriented cDNA was subcloned at the *Hin*dIII/*Apa*I sites of pRc/CMV mammalian expression vector containing neomycin-resistant selection marker (Invitrogen, San Diego, CA).

Sequencing of the cloned α2→6ST cDNA and transfection into COS-1 cells

DNA sequences of the cloned α2→6ST were determined by the dideoxynucleotide chain-termination method (33) using the Sequenase version 2.0 DNA sequencing kit (United States Biochemicals Co., Cleveland, OH) and a Sequi-Gen apparatus (Bio-Rad, Hercules, CA). COS-1 cells were transfected with the cloned α2→6ST by the electroporation method as described (6). The transfected COS-1 cells were selected in the presence of G418 (GIBCO, Gaithersburg, MD) and cloned by the limiting dilution method.

Construction of the staphylococcal protein A-α2→6ST fusion

The cDNA fragment containing nucleotide residues 113–1227 (see Fig. 2) of α2→6ST was prepared by digestion with *Eco*RI and blunted with T4 DNA polymerase. *Eco*RI adaptor (New England Biolab, Beverly, MA)

5′-AATTCCCGGG

GGGCCC-5′

was added to the blunted fragment. The resultant sequence was inserted at the unique *Eco*RI site of pPROTA2 vector (kindly provided by Dr. R. Breathnach) (34) to yield the expression plasmid pPROTA2-α26ST. For control experiment, full-length cDNA of α2→6ST was prepared by digestion with *Bst*XI and subcloned into BstXI site of pCDM8 vector resulting pCDM8–α26ST plasmid. Fifty μg of the each plasmid were transfected into 1×10^7 COS-1 cells, and the conditioned medium was collected after 48 h and processed as described elsewhere (35).

RNA blot analysis

Total cellular RNA was extracted using guanidinium/CsCl method. Poly(A)-RNA was purified using Oligotex-d(T) according to the method described by the supplier (TAKARA, Kyoto, Japan). The amounts of total RNA or poly(A)-RNA were estimated spectrophotometrically. RNA was aliquoted and precipitated in ethanol until use. RNA blot analysis was carried out as described (6) using ^{32}P-labeled full-length α2→6ST cDNA fragment or a neomycin-resistant cDNA fragment.

Sialyltransferase assays

The total cell membranous fractions were prepared as described previously (14), aliquoted, and stored at −80°C until use. CMP-NeuAc:nLc_4Cer α2→3 and α2→6sialyltransferase activities were assayed as described previously (14). In brief, 10 nmol of nLc_4Cer and 2.5–10 μL of CMP-[sialic-4,5,6,7,8,9-^{14}C]NeuAc were added in a microtube and then dried. Cold CMP-NeuAc (4.34–4.80 nmol), sodium cacodylate buffer (pH 6.5, 3.75 μmol), Triton CF-54 (75 μg), and the enzyme preparation were mixed and incubated in a total volume of 25 μL at 37°C. The incorporation of [^{14}C]NeuAc into glycosphingolipid was determined by direct identification of the reaction products on HPTLC plate using BAS 2000 bioimage analyzer (Fuji Film, Tokyo, Japan). The solvent system used in this assay was chloroform–methanol–0.2% $CaCl_2$ 50:50:10 (vol/vol/vol). The activities of CMP-NeuAc:β-galactoside α2→3 and α2→6sialyltransferases were also assayed using nLc_4-PA as an acceptor substrate and HPLC system with μBondasphare C18 column (3.9 × 150 mm) as described (14) with slight modifications for sialyltransferase assay instead of β1→3-*N*-acetylglucosaminyltransferase assay. The catalytic NeuAc transfer activities of α2→6ST onto glycoproteins were assayed using Sephadex G-50 chromatography as described (36).

Analyses of gangliosides

Cells for glycosphingolipid analyses were harvested and stored at −80°C until use. Glycosphingolipids were prepared by the combination of chloroform–methanol extraction and DEAE-Sephadex A-25 column chromatography (37). After mild alkaline treatment, dialysis and lyophilization, ganglioside fractions were separated on HPTLC plate with a solvent system of chloroform–methanol–0.2% $CaCl_2$ 50:50:10(v/v/v). Immunostaining was performed by the method described by Magnani, Smith, and Ginsburg (38) with slight modification (6). Authentic standards were visualized using the resorcinol-HCl spray method followed by heating at 105°C for 5 min.

Treatment of enzyme reaction products with Newcastle disease virus and *A. ureafaciens* neuraminidases

After sialyltransferase assay using purified pPROTA2-α26ST fusion in the presence of CMP-[sialic-4,5,6,7,8,9-^{14}C]NeuAc and nLc_4Cer, the reaction product was subjected to heat inactivation at 60°C for 10 min and NDV neuraminidase treatment as follows. Twenty-five μL of α2→6ST reaction product was mixed with NDV neuraminidase (final 20 mU/mL concentration) or *A. ureafaciens* neuraminidase (40 mU/mL), sodium taurodeoxycholate (2.5 mg/mL), and sodium cacodylate buffer, pH5.5 (300 mM) in a total volume of 100 μL, incubated at 37°C for 1.5 h, purified with SepPak C18 reverse phase cartridge, evaporated under N_2 flow, and separated on HPTLC plate in a solvent system of chloroform–methanol–0.2% $CaCl_2$ 55:50:10 (v/v/v). The radioactive products were visualized by autoradiography and quantitated by BAS 2000 bioimage analyzer. For the control reaction, an aliquot of the product was incubated in the same condition without any neuramini-

dase. For another control reaction, the radioactive authentic standard mixture was prepared using the membranous fraction of human B lymphoid cell line U266, CMP-[sialic-4,5,6,7,8,9-^{14}C]NeuAc, and nLc$_4$Cer, and treated with NDV neuraminidase.

Protein assay and analysis by polyacrylamide gel electrophoresis

Protein was determined by an Amido-Schwarz dye-binding method (39) with bovine serum albumin as a standard. The purified rat liver α2→6ST was analyzed for purity by PAGE containing SDS. The enzyme was denatured by boiling for 5 min in the presence or absence of 2-mercaptoethanol (2-ME), and loaded onto 8% polyacrylamide gel containing 0.1% SDS. After electrophoresis for 1 h at 20 mA, the proteins were visualized by silver staining.

RESULTS

NeuAc transfer activities of purified α2→6ST onto nLc$_4$Cer

The purified α2→6ST, that was assured to be pure using 1 μg of enzyme protein by PAGE analysis followed by silver staining (data not shown), was tested for catalyzing NeuAc transfer to glycolipid, nLc$_4$Cer. As Triton CF-54 at a range of concentration 0.03–0.3% was most effective for detecting the enzyme activities, 0.3% Triton CF-54 was selected as detergent. Subsequently, the time course of the enzyme reaction was analyzed as shown in **Fig. 1A.** The NeuAc transfer to nLc$_4$Cer was catalyzed linearly up to at least 2 h. As the enzyme activities seemed to plateau after 2 h, we stopped the enzyme reactions at 1 h for the subsequent assays. Then we investigated the protein concentration dependency as indicated in Fig. 1B. The purified protein was clearly shown exhibited to have catalytic effect on NeuAc transfer to nLc$_4$Cer in a concentration-dependent manner up to 0.15–0.25 μg protein per each 25 μL assay. Beyond these concentrations, the activities did not increase linearly. Furthermore, the purified α2→6ST was verified to be dependent on the concentration of donor substrate, CMP-NeuAc (K_M value for CMP-NeuAc was 0.05 mM), and of acceptor substrate, nLc$_4$Cer (data not shown).

Acceptor specificity of purified α2→6ST

To test the acceptor specificity of the purified α2→6ST, kinetic constant K_M values of the enzyme against various acceptor substrates were determined under the standard assay conditions (**Table 1**). In this table, K_M value was calculated as mM concentration of a whole molecule, glycoprotein, glycosphingolipid, or oligosaccharide. The concentration of sugar-donor substrate, CMP-[^{14}C]NeuAc, was fixed at 0.15 mM for each assay. While the purified enzyme had K_M values around 0.2 mM for glycoprotein acceptors, ASα1AGP and asialo transferrin, K_M for glycolipid acceptor nLc$_4$Cer was 0.9 mM and slightly higher than those for glycoprotein acceptors. For synthetic substrates, nLc$_4$-PA and Galβ1→4GlcNAc-PA, the enzyme protein had K_M values of 1.5 and 1.3 mM, respectively. However, Galβ1→4Glc-PA was revealed to require higher concentration to get to V_{max} for α2→6ST than nLc$_4$-PA. In addition, Lc$_3$Cer, Lc$_4$Cer, Lc$_4$-PA, and Galβ1→3GlcNAc-PA were shown to be poor acceptors.

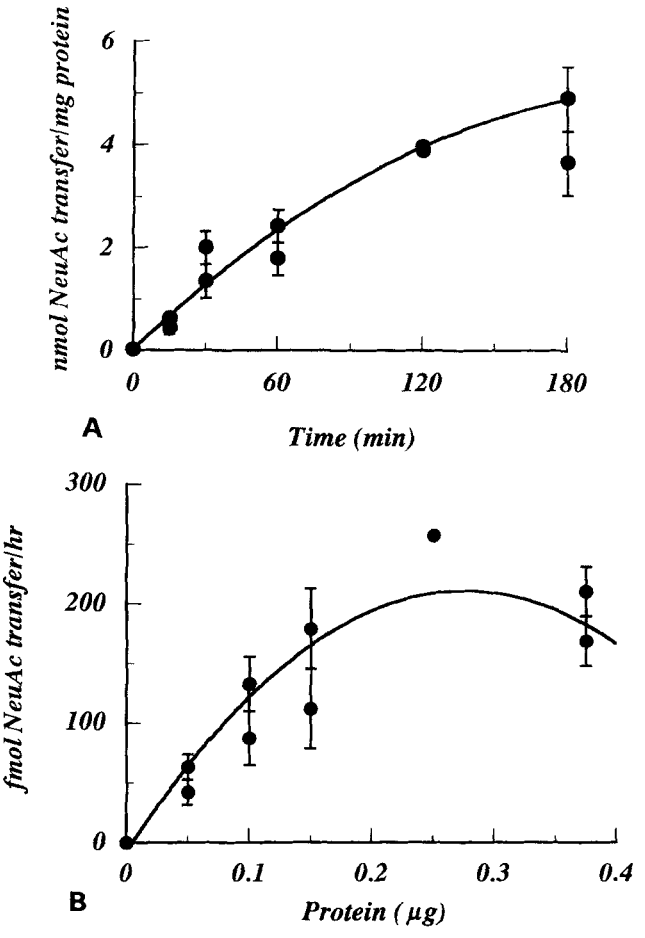

Fig. 1. Enzymatic analyses of rat liver α2→6ST using nLc$_4$Cer as an acceptor. A: Effect of incubation time on the pure α2→6ST. The activities were assayed by incubating the enzyme in the presence of 0.3% Triton CF-54 for various periods of time. B: Dependency on the enzyme protein concentration. The activities were assayed by incubating various amounts of the enzyme for 1 h in the presence of 0.3% Triton CF-54. Each value is the mean ± SE (n = 4) from a representative experiment. Where an error bar is not shown, it lies within the dimensions of the symbol.

Transfection and expression in COS-1 cells of α2→6ST

To exclude the possibility that the catalytic activities of α2→6ST for glycolipid acceptors were due to a mi-

TABLE 1. Apparent K_M values of the rat liver α2→6ST against acceptor substrates

Acceptor	K_M Values[a] of α2 → 6ST
Galβ1→4GlcNAcβ1→3Galβ1→4Glc-PA (nLc$_4$-PA)	1.5
Galβ1→4GlcNAc-PA	1.3
Galβ1→4Glc-PA (Lac-PA)	102
Galβ1→3GlcNAcβ1→3Galβ1→4Glc-PA (Lc$_4$-PA)	[b]
Galβ1→3GlcNAc-PA	[b]
ASα1AGP	0.20
Asialo transferrin	0.16
nLc$_4$Cer	0.91
Lc$_4$Cer	[b]
Lc$_3$Cer	[b]

Kinetic constant K_M values were determined under the standard conditions described in Experimental with a fixed sugar-donar concentration of CMP-[^{14}C]NeuAc at 0.15 mM. Plots of the data in double reciprocal form were constructed and slope and intercepts were determined by the method of least squares.

[a]mM as whole acceptor protein or glycolipid molecules.

[b]Not measurable; the substrates were poor acceptors.

nor contamination of the other α2→6sialyltransferase enzyme protein, we used the mammalian overexpression system for analyzing the sialyltransferase substrate specificity. As a first step, we isolated, from human Burkitt lymphoma cell line Daudi, several cDNA clones that encode α2→6ST protein. Among them, clones #5, #10, and #20 were selected after PCR amplification followed by subcloning to pCRII vector and to pRc/CMV vector. By DNA sequencing, the three clones were examined to have no deletion or frame shift. Further, we found that these three clones were identical to the clone isolated from human placenta (40) rather than the sequence from the same cell line Daudi reported elsewhere (41). **Figure 2** summarizes identities and differences of deduced amino acid sequences of the clones to and from the previously reported sequences. As shown in Fig. 2A, there were three major points where the amino acid substitutions occurred among the several clones. Comparing with the sequences from Daudi previously reported (41), the amino acid of the three clones was lysine instead of leucine at the position 27, there was no deletion at the position 72, and the amino acids were His-Arg at the positions of 72 and 73. Although the clone #20 had arginine substitution for lysine at the position 240, there was no substitution in the other two clones at this position. As indicated in Fig. 2C, there were three additional nucleotide substitutions among the three clones and the previously reported sequences from Daudi. However, these did not affect the amino acid sequences at all. Consequently, we chose the clones #5 and #10 for the transfection and expression studies, as the clone #20 had a substitution in the middle of catalytic domain of the sequence (Fig. 2B).

Using stable transfectants of the cDNA clones #5 and #10, we analyzed expression of α2→6ST in COS-1 cells. As a first step, we characterized the transfected cells by Southern and RNA blot analyses. Southern blot analysis showed that a band additional to those found in the parental COS-1 cells was exhibited in the transfected cells (data not shown). RNA blot analysis revealed that COS-1 cells transfected with #10 clone ligated to pRc/CMV expressed significant and high amount of the single 4.1 kb α2→6ST message (**Fig. 3;** lane 3; arrow A). By contrast, the parental COS-1 cells and the cells transfected with pRc/CMV vector alone did not express the transcript (Fig. 3; lanes 1, 2). For the message of the neomycin-resistant gene, the COS-1 cells transfected with pRc/CMV vector alone and with #10 clone ligated to pRc/CMV expressed neomycin-resistant gene transcripts (Fig. 3; lanes 5, 6; arrow B). The cells transfected with #5 clone ligated to pRc/CMV also expressed α2→6ST message and neomycin-resistant gene message (data not shown). Consequently, it could be thought that exogenous α2→6ST gene was successfully integrated in chromosomes and expressed constitutively.

NeuAc transfer activities of α2→6ST overexpressed in COS-1 cells

Using crude membrane fractions from the transfectants, NeuAc transfer activities on glycoprotein acceptors were initially determined. As shown in **Fig. 4,** the membranous fraction from COS-1 cells transfected with the clone #10 α2→6ST cDNA catalyzed NeuAc transfer to ASα1AGP in a dose-dependent manner. However, the transfectant with pRc/CMV vector alone did not have significant activity. In addition, the COS-1 cell transfectant with the clone #10 α2→6ST cDNA did not catalyze NeuAc transfer to α1AGP.

Subsequently, the NeuAc transfer activities of the transfectants to glycolipid acceptors were examined. Optimal conditions were determined through the sialyltransferase assays using nLc$_4$Cer as an acceptor (data not shown). Detergent and its concentration were selected as Triton CF-54 and at 0.3%, respectively. The assays were done at 37°C for 1 h using 200 μg of protein, 1.5 mM nLc$_4$Cer, and 0.5 mM CMP-[^{14}C]NeuAc. Under these conditions, the activities for producing IV^{6}NeuAc–nLc$_4$Cer were elevated up to 7- to 9-times higher in the transfectants #5 and #10 than in the transfectant with vector alone (**Fig. 5;** columns 3–5). By contrast, α2→3sialyltransferase activities to nLc$_4$Cer did not show significant difference between the transfectants with the clone #10 α2→6ST cDNA and with vector only (Fig. 5; columns 1, 2).

Acceptor specificity of the α2→6ST overexpressed in COS-1 cells

Acceptor specificity of the crude membrane fraction from COS-1 cells transfected with the clone #10 cDNA was analyzed using various possible acceptors. To com-

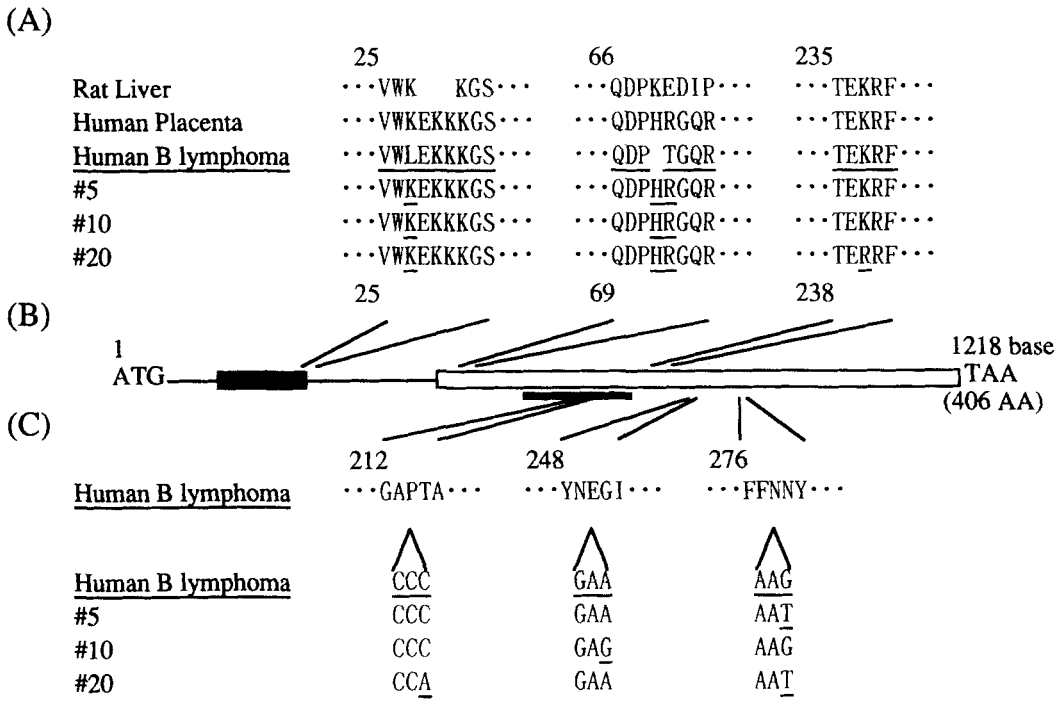

Fig. 2. *Structure and deduced amino acid sequence of α2→6ST cDNA. (A) The partial deduced amino acid sequences of α2→6ST clone #5, #10, and #20 from human Burkitt lymphoma cell line Daudi and the comparison with those from rat liver (22), human placenta (40), and human B lymphoma cell line (41). The numbers at the top and the bottom of the sequences represent the amino acid positions from the N-termini of the rat liver sequence and the clone #20, respectively. (B) Schematic illustration of the cloned human α2→6ST cDNA from Daudi. The numbers at the top of the illustration represent the nucleotide positions. The closed and the open boxes represent the transmembrane and the catalytic domains, respectively. The bar at the bottom of the open box represents the position of the first sialyl motif. (C) The partial deduced amino acid sequences of α2→6ST cDNA from human B lymphoma cell line (41) and the respective nucleotide sequences of the reported and the cloned cDNA, #5, #10, and #20. The numbers at the top of the amino acid sequences represent the positions from the N-terminus of that from the human B lymphoma α2→6ST.*

pare specific activities of α2→6ST on glycoprotein and glycosphingolipid on the basis of possible acceptor concentrations, the relative activity values were calculated as mole NeuAc transfer/mole acceptor oligosaccharides per mg enzyme protein per h, and expressed as ratios to the NeuAc transfer activity to nLc_4Cer (**Table 2**). While Lc_4Cer and Lc_3Cer did not accept NeuAc in α2→6 linkage, nLc_4Cer, nLc_6Cer, and synthetic nLc_4-PA were shown to be good acceptors using α2→6ST overexpressed in COS-1 cells. For glycoprotein acceptor, ASα1AGP was proved to be an 8-fold better acceptor than glycolipids in this overexpression system. As ASα1AGP has various types of possible acceptor sites on its molecule (47 oligosaccharides per one molecule of ASα1AGP), we studied the NeuAc transfer activities on a synthetic neoglycoprotein, nLc_4-HSA, that has only nLc_4Ose on the protein molecule as acceptor sites (15 oligosaccharides per one molecule of nLc_4-HSA). Strikingly, the overexpressed crude enzyme preparation was demonstrated to have greater catalytic activities on nLc_4-HSA than on ASα1AGP as presented in Table 2.

Apparent K_M values of α2→6ST overexpressed in COS-1 cells for acceptor substrates, ASα1AGP and nLc_4Cer, were determined under the standard assay conditions with fixed CMP-[^{14}C]NeuAc concentration at 0.15 mM. In Table 2, K_M values were calculated in two different ways; K_M1, mM as whole acceptor molecules of glycoprotein or glycolipid; K_M2, mM as acceptor oligosaccharide molecules. K_M1 for ASα1AGP was 0.28 mM, and there was little difference between the purified enzyme preparation and the crude enzyme fraction from COS-1 cells transfected with the clone #10 α2→6ST cDNA. Likewise, K_M1 against nLc_4Cer was 0.82 mM, and this value was almost the same as of the purified α2→6ST. However, K_M2 against ASα1AGP and nLc_4-HSA were calculated as 13.1 and 3.3 mM as they had 47 and 15 possible acceptor oligosaccharides on each protein molecule, respectively. Using the K_M2 values, V_{max}/K_M ratios of the α2→6ST overexpressed in COS-1 cells for acceptor substrates were also determined and calculated under the standard assay conditions; the ratio was designated as V_{max}/K_M2 ratio in Table 2. The crude membranous fraction from the transfectants revealed that α2→6ST had 0.59 and 0.75 as V_{max}/K_M ratios for nLc_4Cer and nLc_4-PA, respectively. Although the ratios for glycolipid acceptors were lower than those for

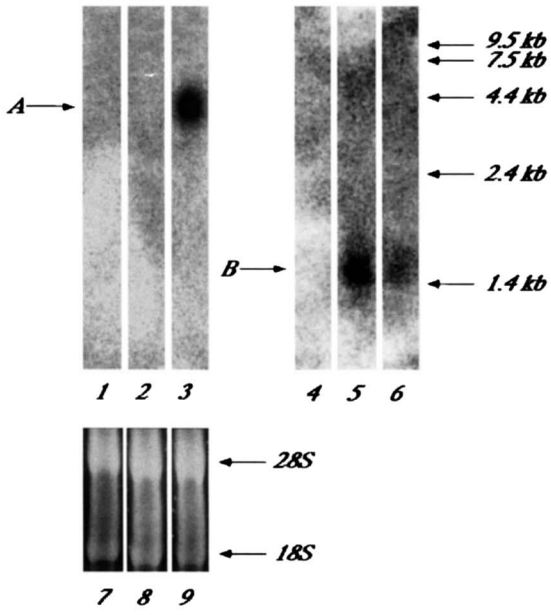

Fig. 3. RNA blot analyses of the COS-1 cells transfected with α2→6ST cDNA. Each 20 μg total RNA from COS-1 cells (lanes 1, 4, and 7), COS-1 cells transfected with the vector pRc/CMV alone (lanes 2, 5, and 8), and from COS-1 cells transfected with the α2→6ST cDNA-containing plasmid #10 (lanes 3, 6, and 9) was electrophoresed in 0.75% agarose gel, blotted to nylon membrane, Biodyne B (Nippon Genetics, Tokyo, Japan), hybridized with ^{32}P-labeled α2→6ST cDNA, and detected with autoradiography (lanes 1, 2, and 3). The membrane was rehybridized with ^{32}P-labeled neomycin resistant cDNA fragment (lanes 4, 5, and 6). The ribosomal RNA was stained with ethidium bromide (lanes 7, 8, and 9). The arrows indicate the positions of RNA markers 9.5, 7.5, 4.4, 2.4, and 1.4 kb, and 28S and 18S ribosomal RNA bands. Arrows A and B indicate the positions of the messages from the transfected cDNA, α2→6ST and neomycin-resistant selection marker, respectively.

glycoprotein acceptors ASα1AGP (0.95) and nLc_4-HSA (5.12), the α2→6ST had substantial activities for glycolipid acceptors as well as for glycoproteins on the basis of acceptor oligosaccharide concentration levels.

Product identification of NeuAcα2→6Gal structures

Figure 6A and B demonstrate the results of product identification on HPTLC plate using α2→6ST overexpressed in COS-1 cells as crude enzyme preparation and nLc_4Cer as an acceptor substrate. While the transfected cells with vector alone catalyzed NeuAc transfer to nLc_4Cer in only α2→3 linkage (Fig. 6A; lane 1), the transfectant with the clone #10 α2→6ST cDNA was clearly demonstrated to generate IV^{6}NeuAc–nLc_4Cer along with IV^{3}NeuAc–nLc_4Cer (Fig. 6A; lane 2). The structure of IV^{6}NeuAc–nLc_4Cer was not only estimated by the mobility on HPTLC plate comparing with the authentic standard, but also proved to have NeuAcα2→6Gal structure at the non-reducing end by TLC-immunostaining using a mAb antibody 1B9 (Fig.

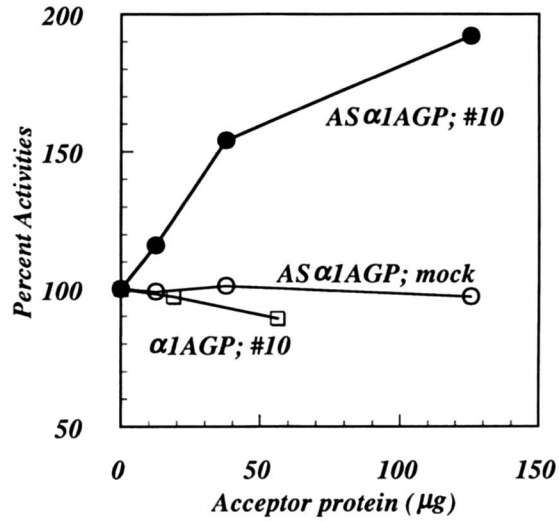

Fig. 4. Relative activities of the transfected α2→6ST against glycoprotein acceptors. The sialyltransferase activities were assayed by incubating the crude membranous enzyme preparations (200 μg protein per assay) from COS-1 cells transfected with the clone #10 and with the vector only (designated as mock) in the presence of ASα1AGP or α1AGP. The detergent and sugar donor substrate concentrations were Triton CF-54 at 0.3% and CMP-NeuAc at 0.5 mM, respectively. The values were expressed as relative activities comparing with those without acceptor protein (100% = 19.9 nmol/mg protein/h) and means of two determinations. Similar results were obtained in replicate assays using replicated cell cultures.

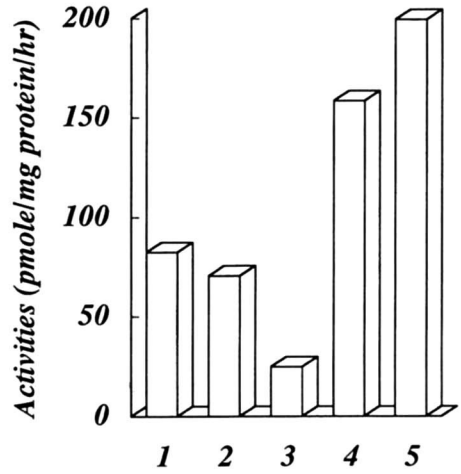

Fig. 5. Activities of the transfected α2→6ST against glycolipid acceptors. The sialyltransferase activities were assayed by incubating the crude membranous enzyme preparations from COS-1 cells transfected with the clone #5, #10, and the control vector (mock transfection) in the presence of glycolipid acceptor nLc_4Cer. The catalytic activity on nLc_4Cer was determined by direct identification of the reaction products on HPTLC plate using BAS 2000 bioimage analyzer. Column 1; α2→3sialyltransferase activities from the mock transfection, column 2; α2→3sialyltransferase activities from the clone #10, column 3; α2→6sialyltransferase activities from the mock, column 4; α2→6sialyltransferase activities from the clone #5, column 5; α2→6sialyltransferase activities from the clone #10.

TABLE 2. Acceptor specificity of the enzyme fraction from COS-1 cells transfected with cDNA encoding α2→6ST

Acceptor	Relative Activities	K_M1^a	K_M2^b	V_{max}^c	V_{max}/K_M2 Ratio
ASα1AGP	8.29	0.28	13.1	12.4	0.95
nLc$_4$-HSA	11.3	0.22	3.30	16.9	5.12
nLc$_4$Cer	1	0.82	0.82	0.48	0.59
nLc$_6$Cer	1.11	n.d.	n.d.	n.d.	n.d.
nLc$_4$-PA	1.85	1.1	1.1	0.82	0.75
Lc$_4$Cer	<0.01	n.d.	n.d.	n.d.	n.d.
Lc$_3$Cer	<0.01	n.d.	n.d.	n.d.	n.d.

Activities of the transfected α2→6ST into COS-1 cells in the optimal condition were measured as described in Experimental section. The relative activities were calculated comparing each activity as mole NeuAc transfer/mole acceptor oligosaccharides/mg enzyme protein/h and expressed as ratios to the NeuAc transfer activities to nLc$_4$Cer. The units of K_M are expressed in two ways; mM as a whole acceptor molecule (K_M1) and mM as acceptor oligosaccharide molecules (K_M2). The V_{max} unit is expressed as nmole NeuAc transfer/mg protein/h and the data V_{max}/K_M2 ratio is presented. Kinetic constants were determined by plotting the data in double reciprocal form, and by calculating slope and intercepts by the method of least squares; n.d., not determined.

[a] mM as whole acceptor protein or glycolipid molecules.
[b] mM as acceptor oligosaccharide molecules.
[c] nmole NeuAc transfer/mg enzyme protein/h.

6B, lane 3). The specificity of 1B9 mAb has been characterized to be the terminal NeuAcα2→6Galβ1→(32). These results suggested that the NeuAcα2→6Gal-bearing reaction product observed in the assay mixtures containing nLc$_4$Cer was due to the action through α2→6ST cDNA transfected into COS-1 cells.

Analyses of NeuAcα2→6Gal structure-containing gangliosides in the transfected COS-1 cells

To further confirm the effect of the α2→6ST cDNA action on the transfected cells, we characterized the COS-1 cells transfected with the cDNA in the in situ situation. The NeuAcα2→6Gal structure-containing gangliosides in the transfectants were analyzed by TLC-immunostaining method using a mAb antibody 1B9 (38). As shown in **Fig. 7,** an 1B9-reactive ganglioside in the transfected COS-1 cells with the clone #5 and #10 of α2→6ST cDNA was clearly detected by the analysis (lanes 2 and 3), while those in the mock transfectant were faint (lane 1). Compared to the positions of the authentic IV^{6}NeuAc–nLc$_4$Cer, the structure of the main 1B9-reactive ganglioside in the transfected cells was not IV^{6}NeuAc–nLc$_4$Cer. The TLC-immunostaining data indicated that at least a ganglioside containing the terminal NeuAcα2→6Gal structure was generated by the action of α2→6ST cDNA transfected in COS-1 cells in the in situ situation.

Analyses using a soluble staphylococcal protein A-α2→6ST fusion protein

To exclude the possible contamination of the other sialyltransferase or involvement of a *trans*-acting molecule that would determine α2→6ST activities on glycosphingolipids by interaction with an endogenous gene, transcript, or protein, we fused the catalytic and stem domain of the predicted polypeptide to a secreted form of the IgG binding domain of staphylococcal protein A. Conditioned media prepared from COS-1 cells transfected with pPROTA2-α26ST or with pCDM8-α26ST contained substantial α2→6ST activities on glycosphingolipids as shown in **Table 3.** However, the released activity generated by pPROTA2-α26ST was specifically retained by the IgG-Sepharose, whereas the activity by pCDM8-α26ST was not bound. These results show that the protein encoded by the α2→6ST directly catalyzes NeuAc transfer onto glycosphingolipids.

Using the soluble α2→6ST fusion, sequence specific NDV neuraminidase (42), and sequence nonspecific *A. ureafaciens* neuraminidase, the enzyme product was further identified on the HPTLC plate as shown in Fig. 6C (43). While NeuAcα2→3Gal-specific NDV neuraminidase could not catalyze the purified pPROTA2-α26ST reaction product (lane 7), *A. ureafaciens* neuraminidase hydrolyzed a significant amount of the product (lane 8); the remaining products were only 20% of the mock treatment (lane 6) and 13% of NDV neuraminidase treatment. As NDV neuraminidase could hydrolyze the radiolabeled authentic standard IV^{3}NeuAc–nLc$_4$Cer (lanes 9, 10), the negative result in the lane 7 was thought to be significant. Consequently, together with the results using NeuAcα2→6Galβ1→specific mAb 1B9 (Fig. 6; panel B), the linkage of sialyl-paragloboside formed by the cloned sialyltransferase was considered as NeuAcα2→6Gal instead of NeuAcα2→3Gal.

DISCUSSION

To date over 10 sialyltransferases have been cloned from various cells or tissues. Among them, Galβ1→3-GalNAc α2→3sialyltransferase (44), GalNAc α2→6sialyltransferase (45), and Galβ1→3GalNAc (GalNAc specific) α2→6sialyltransferase (46) were reported to be *O*-glycan specific or preferable. While CMP-NeuAc: GM3 α2→8sialyltransferase (GD3 synthase) (47) is ganglioside specific α2→8sialyltransferase, polysialyltransferase-1 from hamster (48) and a developmentally regulated member of the sialyltransferase family (STX) (49, 50) were reported to catalyze NeuAc transfer onto polysialic acid and onto *N*-linked oligosaccharides in α2→8 linkage, respectively. On the other hand, Galβ1→3/1→4GlcNAc α2→3sialyltransferase (51), Galβ1→3GalNAc/Galβ1→4GlcNAc α2→3sialyltransferase (52), and Galβ1→3GalNAc α2→3sialyltransferase (53) catalyze their reaction both on glycoprotein and on glycosphingolipids. The latter two, however, were demon-

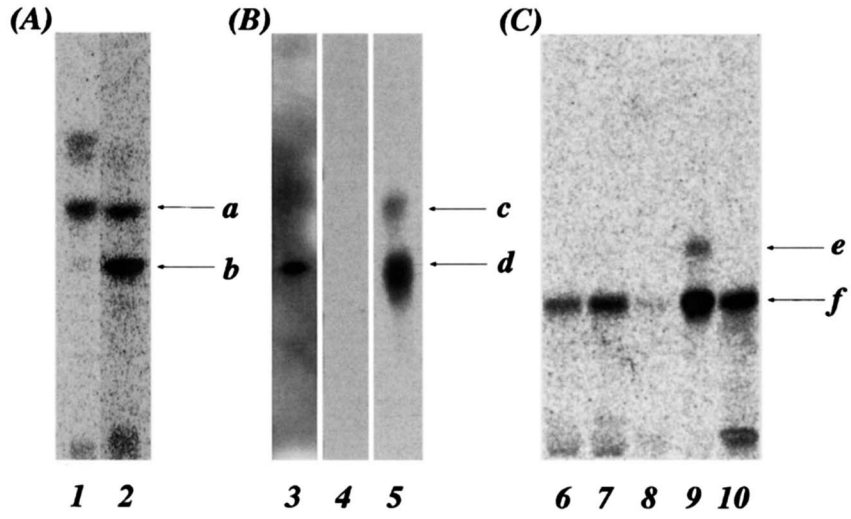

Fig. 6. Product identification of α2→6ST from the transfected COS-1 cells. (A) The reaction products from the assay using the crude membranous enzyme preparations form COS-1 cells transfected with the control vector (lane 1) or with the clone #10 (lane 2) in the presence of glycolipid acceptor nLc_4Cer were purified by reverse phase column chromatography and separated on the HPTLC plate using the solvent chloroform–methanol–0.2% $CaCl_2$ 50:50:10 (v/v/v) (see Experimental section for the details). The arrows indicate the positions of IV^3NeuAc-$nLcOse_4Cer$ (a) and IV^6NeuAc-$nLcOse_4Cer$ (b). (B) The reaction products from the several assay mixtures using the enzyme preparation from COS-1 cells transfected with the clone #10 in the presence of nLc_4Cer were prepared, separated on the HPTLC plate using the same solvent system, and detected by TLC-immunostaining method using 1B9 mAb (lane 3) and an irrelevant mAb against human IgG (lane 4). Lane 5; authentic standards IV^3NeuAc-$nLcOse_4Cer$ (arrow c) and IV^6NeuAc-$nLcOse_4Cer$ (arrow d) visualized by resorcinol-HCl spray. (C) The reaction products from the assay using the purified pPROTA2-α26ST fusion protein in the presence of radioactive sugar nucleotide donor and acceptor nLc_4Cer were treated with NDV and *A. ureafaciens* neuraminidases, purified by SepPak C18 reverse phase cartridge, evaporated, separated on the HPTLC plate using the solvent system of chloroform–methanol–0.2% $CaCl_2$ 55:50:10 (v/v/v), and detected by autoradiography. Lanes 6, 7, and 8 are mock treatment, NDV neuraminidase treatment, and *A. ureafaciens* neuraminidase treatment, respectively. Lane 9 is the authentic standard mixture [sialic-4,5,6,7,8,9-^{14}C]IV^3NeuAc-$nLcOse_4Cer$ (arrow e) and [sialic-4,5,6,7,8,9-^{14}C]IV^6NeuAc-$nLcOse_4Cer$ (arrow f). Lane 10 is the NDV neuraminidase-treated authentic standard mixture.

strated to preferentially sialylate the terminal Gal of glycosphingolipids in α2→3 linkage rather than of glycoproteins.

By contrast, it has been still uncertain whether glycosphingolipids could be good acceptors of the α2→6ST (22) or whether there could be another different α2→6sialyltransferase specific for glycosphingolipids. In our present study, cDNAs, that encode α2→6ST, were cloned from human Burkitt lymphoma cell line Daudi and sequenced. Although our clones had sequence heterogeneity different from that reported elsewhere (41), the transfected COS-1 cells were revealed to express α2→6ST activities not only on glycoprotein but also on glycosphingolipid acceptors. According to the elevated activities, the acidic glycolipid fraction in the transfectants contained a greater amount of NeuAcα2→6Gal-containing component than the fraction from the mock transfectants. Our results from the analysis in this in situ situation suggested that the in vitro observations found in kinetic experiments were not due to a minor contamination of the other α2→6sialyltransferases specific for glycosphingolipids or due to an artifactual specificity of the enzyme. Instead, the observations can be thought as the results of the action of α2→6ST cDNA transfected into the cells. Furthermore, staphylococcal protein A-α2→6ST fusion protein was shown to exhibit substantial activity on nLc_4Cer. This indicates that the protein encoded by the α2→6ST directly catalyzes NeuAc transfer onto glycosphingolipids instead of in a *trans*-acting manner.

For sequence heterogeneity of α2→6ST, two transcripts have been identified in cells with a mature B-cell phenotype and in placenta (40, 41). These were reported to differ considerably in a part of their 5′ untranslated regions (54). However, we cloned and sequenced three cDNA transcripts from Burkitt lymphoma cell line Daudi and showed that the sequences were the types of placenta rather than those of B-cells in spite of the cloning source. As shown in Fig. 2, all three clones had Lys instead of Leu at the position of 27 and His-Arg instead of Thr at the position of 72–73. The heterogeneity at the position 240 was supposed to be due to an error of AmpliTaq DNA polymerase during PCR reaction, as only clone #20 had Arg instead of Lys. On the other hand, the other heterogeneity at the positions of 27 and 72–73 were not due to PCR er-

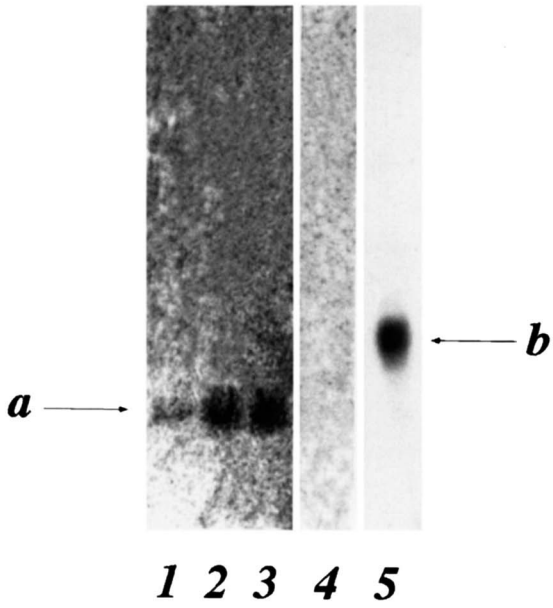

Fig. 7. Analyses of glycosphingolipids containing NeuAcα2→6Gal sequences in the α2→6ST-transfected COS-1 cells. Ganglioside fractions were prepared from the clone #5 and #10-transfected COS-1 cells and the mock transfected cells. One μg of ganglioside was spotted in each lane, separated on HPTLC plate, and stained sequentially with 1B9 monoclonal Ab or an irrelevant mAb against human IgG, with rabbit anti-mouse IgM Ab, and with ^{125}I-labeled protein A. Lane 1; the mock transfected COS-1 cells, lane 2; the clone #5-transfected cells, lanes 3–4; the clone #10-transfected cells. Lanes 1–3; stained with mAb 1B9, lane 4; stained with mAb against human IgG. Arrow a indicates the position of the detected signals. Lane 5; the authentic standard IV^{6}NeuAc-nLc$_4$Cer (arrow b) visualized by the resorcinol-HCl spray method.

rors, because these sequences were in good agreement among the three clones. It would be of interest to investigate the reason and mechanism why our cell line Daudi generates the placenta type instead of B-cell type transcript.

α2→6ST has been demonstrated to play crucial roles generating leukocyte cell surface-differentiation antigens; CDw75 and CD76 (16). In addition, CD76 was reported to be an α2→6 sialylated lacto-series motif found on glycolipids (20). Our current results support that the same α2→6ST could possibly generate cell surface carbohydrate epitopes CDw75 on glycoproteins and CD76 on glycosphingolipids. In fact, our recent observations using human myelogenous leukemia cell line HL-60 cells revealed the roles of α2→6ST during cell differentiation. *i)* The activities of the α2→6ST are up-regulated during monocytic and granulocytic differentiation (14) *ii)* The message of the α2→6ST is also up-regulated during differentiation into both lineages (M. Nakamura, A. Tsunoda, Y. Furukawa, T. Kudo, H. Narimatsu, and M. Saito, unpublished results). *iii*) Together with the up- and down-regulations of the most upstream glycosyltransferases in the total metabolic flow of glycosphingolipid biosynthesis (14) the α2→6ST generates CDw75-reacting glycoprotein in monocytic differentiation and CD76-reacting glycosphingolipids in granulocytic differentiation (M. Nakamura, A. Tsunoda, Y. Furukawa, T. Kudo, H. Narimatsu, and M. Saito, unpublished work). It is not yet uncertain whether the location of CDw75-carbohydrate determinant is on *O*-glycans or *N*-glycans. However, our preliminary study implies that CDw75 epitope exists, at least in part, on *O*-glycans (M. Nakamura, A. Tsunoda, Y. Furukawa, T. Kudo, H. Narimatsu, and M. Saito, unpublished results). This suggests that the α2→6ST could utilize *O*-glycans as acceptors as well as *N*-glycans and glycosphingolipids. However, the precise and further elucidation is required.

TABLE 3. Affinity chromatographical analyses of soluble staphylococcal protein A-α2→6ST fusion released from transfected COS-1 cells

	NeuAc Transfer Activity to nLc$_4$Cer					
	IgG-Sepharose			Sepharose		
Vector	Applied	Sap	Bound	Applied	Sap	Bound
	pmol/h/10 mL culture medium					
pCDM8-α26ST	5.8	4.9	n.d.	5.8	5.4	n.d.
pPROTA2-α26ST	20.3	n.d.	9.2	20.3	21.1	n.d.

COS-1 cells were transfected with pCDM8-α26ST or pPROTA2-α26ST and culture media were subjected to chromatography on IgG-Sepharose or Sepharose. Catalytic activity of NeuAc transfer onto nLc$_4$Cer was then determined using the matrix supernatants (Sap) and the washed matrices (Bound). Assay conditions were the same as Fig. 5; n.d., no detectable activity.

Synthetic neoglycoprotein nLc$_4$-HSA, that has only the same terminal oligosaccharide moieties as nLc$_4$Cer and nLc$_6$Cer, accepted more NeuAc in α2→6 linkage than native glycosphingolipid acceptors and synthetic nLc$_4$-PA (Table 2). At least for the α2→6ST, glycoproteins can therefore be thought of as better acceptors than glycosphingolipids. As conformation of a protein moiety was supposed to influence the structure of their sugar chains and the activities of glycosyltransferases that act on the sugar chains (55), some conformational influence of the protein moieties or ceramides may reflect the activity preference. However, it is possible that we have not so far obtained an ideal and optimal condition for glycosyltransferase assay using glycosphingolipids as acceptors or there could be an additional component, such as an activator protein for glycosidases (56), for the complete enzymatic function of the α2→6sialyltransferase. To clarify these points, further studies would be required. Although we do not exclude a possibility that there could be another α2→6sialyltransferase for glycosphingolipids distinct from the α2→6ST used in this study, at least the α2→6ST gene from Daudi was suggested to be one of the responsible sialyltransferase enzymes for generating NeuAcα2→6Galβ1→4GlcNAc sequence on glycosphingolipids.

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