

## Genes Modulated by Expression of GD3 Synthase in Chinese Hamster Ovary Cells

EVIDENCE THAT THE *Tis21* GENE IS INVOLVED IN THE INDUCTION OF GD3 9-O-ACETYLATION\*

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**9-O-Acetylation is a common sialic acid modification, expressed in a developmentally regulated and tissue/cell type-specific manner. The relevant 9-O-acetyltransferase(s) have not been isolated or cloned; nor have mechanisms for their regulation been elucidated. We previously showed that transfection of the GD3 synthase (ST8Sia-I) gene into Chinese hamster ovary (CHO)-K1 cells gave expression of not only the disialoganglioside GD3 but also 9-O-acetyl-GD3. We now use differential display PCR between wild type CHO-K1 cells and clones stably expressing GD3 synthase (CHO-GD3 cells) to detect any increased expression of other genes and explore the possible induction of a 9-O-acetyltransferase. The four CHO mRNAs showing major up-regulation were homologous to VCAM-1, *Tis21*, the KC-protein-like protein, and a functionally unknown type II transmembrane protein. A moderate increase in expression of the *Fx1C1* and *SPR-1* genes was also seen. Interestingly, these are different from genes observed by others to be up-regulated after transfection of GD3 synthase into a neuroblastoma cell line. We also isolated a CHO-GD3 mutant lacking 9-O-acetyl-GD3 following chemical mutagenesis (CHO-GD3-OAc<sup>-</sup>). Analysis of the above differential display PCR-derived genes in these cells showed that expression of *Tis21* was selectively reduced. Transfection of a mouse *Tis21* cDNA into the CHO-GD3-OAc<sup>-</sup> mutant cells restored 9-O-acetyl-GD3 expression. Since the only major gangliosides expressed by CHO-GD3 cells are GD3 and 9-O-acetyl-GD3 (in addition to GM3, the predominant ganglioside type in wild-type CHO-K1 cells), we conclude that GD3 enhances its own 9-O-acetylation via induction of *Tis21*. This is the first known nuclear inducible factor for 9-O-acetylation and also the first proof that 9-O-acetylation can be directly regulated by GD3 synthase. Finally, transfection of CHO-GD3-OAc<sup>-</sup> mutant cells with ST6Gal-I induced 9-O-acetylation specifically on sialylated *N*-glycans, in a manner similar to wild-type cells. This indicates separate machineries for 9-O-acetylation on  $\alpha$ 2-8-linked sialic acids of gangliosides and on  $\alpha$ 2-6-linked sialic acids on *N*-glycans.**

Sialic acids are a family of 9-carbon carboxylated monosaccharides typically located at the termini of mammalian cell surface sugar chains on both glycoproteins and glycolipids. *N*-acetylneuraminic acid, the most common sialic acid, is subjected to various modifications *in vivo* (1–5). One of the most prevalent modifications is *O*-acetylation of the hydroxyl group at the 9-carbon position. This modification is known to reduce or abolish the recognition of sialic acid residues by sialidases (2, 4, 6, 7), by certain sialic acid-binding lectins like Siglecs (4, 5, 8–10), and by several viral recognition proteins (4, 5, 11, 12). Conversely, other viruses require 9-*O*-acetylation for recognition of their target cells (4, 5, 13–16). It is also known that 9-*O*-acetylation is regulated during development and aberrantly expressed in melanomas and basal cell carcinomas (17–20). These findings indicate diverse physiological and pathological roles for 9-*O*-acetylation of sialic acid residues.

*O*-Acetyl groups can be added to the 7- and/or 9-position of sialic acids, with the former migrating to the 9-position, either spontaneously under physiological conditions (21, 22) or under the influence of a specific migrase enzyme (23). In the previous studies, we and others showed that 9(7)-*O*-acetylation is an acetyl-CoA-dependent enzymatic reaction (24–28) that appears to be localized in the *trans*-Golgi apparatus (26, 27, 29, 30) and is presumably catalyzed by a sialic acid-specific 9(7)-*O*-acetyltransferase. Available evidence also suggests that there are multiple distinct *O*-acetyltransferases responsible for *O*-acetylating sialic acids attached to glycans in different linkages and possibly for different classes of glycan chains. For example, transfection of a cDNA encoding CMP-Sia:Gal $\beta$ 1-4GlcNAc $\alpha$ 2-6-sialyltransferase (ST6Gal-I)<sup>1</sup> into Chinese hamster ovary (CHO)-K1 cells (which normally express only  $\alpha$ 2-3-linked sialic acids) induced the expression of 9-*O*-acetyl groups only on the newly appearing  $\alpha$ 2-6-linked sialic acids of *N*-glycans (31). Likewise, transfection of GD3 synthase (CMP-Sia:GM3  $\alpha$ 2-8-sialyltransferase (ST8Sia-I)) into CHO-K1 cells resulted in generation of 9-*O*-acetyl groups only on  $\alpha$ 2-8-linked sialic acids of the newly synthesized disialoganglioside GD3 (31). In contrast,

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<sup>1</sup> The abbreviations used are: ST6Gal-I, CMP-Sia:Gal $\beta$ 1-4GlcNAc $\alpha$ 2-6-sialyltransferase; CD22-Fc, soluble chimeric CD22 conjugated with IgG1 Fc domain; CHE-Fc, soluble chimeric influenza C hemagglutinin esterase fused to IgG1 Fc domain; CHE-FcD, diisopropyl fluorophosphate-treated CHE-Fc; CHO, Chinese hamster ovary; CHO-GD3 cells, CHO-K1 cells stably transfected with GD3 synthase gene; CHO-GD3-OAc<sup>-</sup>, 9-*O*-acetyl-GD3-deficient CHO-GD3 mutant cell derived from CHO-GD3 cells; DD-PCR, differential display PCR; EMS, ethylmethane sulfonate; RT-PCR, reverse transcriptase-mediated PCR; ST8Sia-I, GD3 synthase or CMP-Sia:GM3  $\alpha$ 2-8-sialyltransferase; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; VCAM-1, vascular cell adhesion molecule-1. Ganglioside nomenclature is based on the system of Svennerholm (95).

no 9-O-acetyl sialic acids were detected upon transfection of CMP-Sia:Gal $\beta$ 1-3(4)GlcNAc  $\alpha$ 2-3 sialyltransferase into CHO-K1 cells (31). On the other hand, regulated 9-O-acetylation on murine erythroleukemia cells and on murine T cells appears to be on mucin-like molecules, presumably carried on O-glycans (32, 33).

Many groups have attempted to isolate or clone the 9-O-acetyltransferases responsible for these phenomena, with no success so far. The 9-O-acetyltransferase activity in most systems is very sensitive to solubilization, and thus, direct purification has proven difficult (27, 28, 34). Heterologous cell-cDNA library pairs were applied by several investigators to the attempted expression cloning of a 9-O-acetyltransferase cDNA in COS cells (35-37). However, whereas several candidate genes were isolated, neither a 9-O-acetyltransferase nor a biologically significant inducer for 9-O-acetylation was eventually defined. This suggests that the 9-O-acetyltransferase could be a complex of multiple gene products, not amenable to standard expression cloning. This would fit with our proposal in rat liver Golgi that the acetyl group is transferred to luminal sialic acids via a transmembrane acetyl transfer reaction (26, 34).

The induction of 9-O-acetyl sialic acid by transfection of ST8Sia-I (GD3 synthase) into wild CHO-K1 cells described above can be explained either by the up-regulation of the 9-O-acetyltransferase gene in the presence of this sialyltransferase or by the preexisting expression of an  $\alpha$ 2-8 linkage-specific 9-O-acetyltransferase gene in CHO-K1 cells. If the former is true, analysis of gene expression differences between wild-type CHO-K1 and sialyltransferase gene-transfected CHO cells offers the possibility of detecting the putative 9-O-acetyltransferase gene and/or biologically significant inducers of 9-O-acetylation. We have therefore performed differential display PCR (DD-PCR) between wild type CHO-K1 cells and CHO-K1 cells stably transfected with ST8Sia-I (CHO-GD3 cells). In doing so, we also intended to detect other interesting genes up-regulated by the expression of ST8Sia-I. Such genes might be of interest regarding the induction of 9-O-acetylation or the involvement of GD3 and/or GD3 synthase in biological functions such as differentiation, tissue organization, and regeneration (38-47), tumor biology (19, 20, 48, 49), and apoptosis (50-56). Using this approach and a 9-O-acetyl-GD3-deficient CHO-GD3 mutant cell (CHO-GD3-OAc<sup>-</sup>), we present evidence for a novel pathway in which GD3 induces its own 9-O-acetylation via up-regulation of Tis21, a member of an antiproliferative protein family.

#### EXPERIMENTAL PROCEDURES

**Materials**—Unless otherwise stated, reagents were purchased from Fisher or Sigma, and all oligonucleotides were from Invitrogen.

**Cell Culture**—CHO-K1 cells were obtained from the American Type Culture Collection (ATCC CCL61). All CHO cell lines were grown under 5% CO<sub>2</sub> and 100% relative humidity in  $\alpha$ -minimum essential medium supplemented with 10% (v/v) fetal bovine serum.

**Antibodies and Probes**—The mouse IgG3 anti-GD3 monoclonal antibody R24 (57) was purified from hybridoma supernatant using Protein A-Sepharose as previously described (58). Biotinylated R24 was generated by incubating 20  $\mu$ g of the antibody with 4  $\mu$ g of EZ-link sulfo-*N*-hydroxysuccinimide-biotin (Pierce) in 0.1 M NaHCO<sub>3</sub> (pH 8.3) for 2 h at 4 °C, followed by dialysis against phosphate-buffered saline to remove the excess reagent. The mouse IgG3 anti-9-O-acetyl-GD3 monoclonal antibody 27A (59) was kindly provided by Dr. M. Farquhar (University of California, San Diego) and was used as hybridoma supernatant, or it was purified from supernatant using Protein A-Sepharose (Amersham Biosciences) following the manufacturer's instructions. FITC-conjugated 27A was generated using a Fluorotag FITC Conjugation Kit (Sigma) following the manufacturer's instructions. A recombinant soluble chimera of influenza C hemagglutinin-esterase fused to the Fc region of human IgG1 (CHE-Fc) (60) was produced by stably transfected HEK-293 cells. These cells were also adapted to a protein-free medium, CHO-S-SFM II, and generously provided by Dr. Pascal Crottet (Uni-

versity of Basel) (61). CHE-FcD, the esterase-inactive form of CHE-Fc (60), was prepared by treatment with diisopropyl fluorophosphate as previously described (60). Conditioned medium was collected and concentrated to 4  $\mu$ g/ml protein concentration. For each staining, 150  $\mu$ l of CHE-FcD-containing medium was precomplexed with phycoerythrin-conjugated anti-human IgG1 at a 1:15 dilution (preoptimized for this batch of antibody) in the dark for 2 h at 4 °C. The antibodies were then preabsorbed with CHO-K1 wild type cells to eliminate nonspecific cell surface binding. Samples were stained with these preabsorbed CHE-FcD preparations for 2 h at 4 °C and analyzed by flow cytometry. CD22-Fc, the soluble chimera of the extracellular domain of CD22 fused to the human IgG1 Fc domain, was purified and used at 5  $\mu$ g/ml concentration for cell staining, as previously described (62).

**Construction of CHO-K1 Cells Stably Expressing GD3 and 9-O-Acetyl-GD3**—The expression vector carrying ST8Sia-I for transient transfection has been reported (31). Stable expression of the same cDNA was obtained by transfecting a pcDNA3-based construct into parental CHO-K1 cells using the LipofectAMINE reagent (Invitrogen) and selected with G418. After 68 h, cells were stained with antibody 27A as described below, and individual positive cells were sorted into 96-well plates by fluorescence-activated cell sorting (FACS) using a FACStar unit (Becton Dickinson). The levels of 9-O-acetyl-GD3 expression of individual clones were analyzed by flow-cytometric analysis as described below. One clone that showed high and stable expression of 9-O-acetyl-GD3 (named CHO-GD3) was used for further experiments.

**Generation and Isolation of a Mutant CHO-GD3 Clone Lacking 9-O-Acetylation on GD3**—The cloned CHO-GD3 cell line (stably transfected with ST8Sia-I) was incubated in  $\alpha$ -minimum essential medium containing 450  $\mu$ g/ml ethylmethane sulfonate (EMS; Sigma) for 16 h in a 37 °C incubator. The concentration of EMS was determined in pilot experiments in which the frequency of ouabain resistance was monitored, exactly as previously described (63). Cells were allowed to recover for 72 h in regular medium. Mutagenized cells were then stained with FITC-conjugated 27A antibody and with biotinylated R24 antibody followed by streptavidin-Cychrome (Pharmingen). Cells that stained positive for R24 and negative for 27A were isolated on a FACStar unit (Becton Dickinson). Individual clones were analyzed by flow cytometry as described below. One clone that showed stable GD3 expression (R24-positive) without detectable 9-O-acetylation (27A-negative) was expanded and named CHO-GD3-OAc<sup>-</sup>.

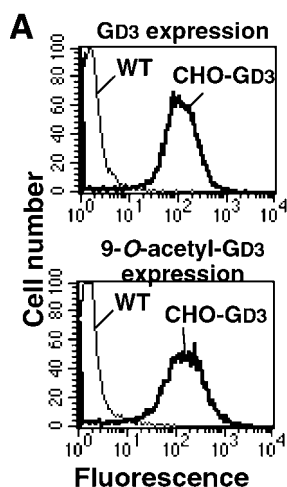
**DD-PCR**—Total RNA was extracted from CHO-K1 wild type and CHO-GD3, respectively, using RNeasy minikit (Qiagen) and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and oligo(dT<sub>11</sub>N) (where N represents A, C, or G) reverse primers (GenHunter) according to the manufacturer's instructions. The resultant first strand cDNA was amplified by PCR using 240 primer pairs of 80 arbitrary forward primers and three oligo(dT<sub>11</sub>N) primer sets (where N represents A, C, or G; GenHunter) and Amplitaq polymerase (PerkinElmer Life Sciences). PCR products were labeled by [<sup>33</sup>P]dATP (PerkinElmer Life Sciences) contained in the PCR mixture. The <sup>33</sup>P-labeled PCR products were resolved on 6% acrylamide gels followed by extraction of the products that were specifically enhanced in CHO-GD3 cells. The isolated products were subcloned into a pCR2.1 TOPO vector (Invitrogen) and sequenced on both strands using M13 primers and an automated DNA sequencer (model 373A; PerkinElmer Life Sciences).

**Reverse Transcriptase-mediated PCR (RT-PCR)**—First strand cDNA was synthesized from total RNA of CHO-K1 wild type, CHO-GD3, and CHO-GD3-OAc<sup>-</sup> using oligo(dT) primer and Superscript II (Invitrogen). PCR was performed using Amplitaq polymerase (PerkinElmer Life Sciences) and gene-specific primers for each DD-PCR product. In addition, the expression of the Chinese hamster isopentenyl-diphosphate: dimethylallyl diphosphate isomerase gene (GenBank™ accession number AF003836) was employed as a control. The RT-PCR products were subjected to electrophoresis on a 1.5% agarose gel.

**Plasmid Construction**—A mouse Tis21 open reading frame was prepared by PCR from a mouse 5'-expressed sequence tag clone (accession number BG973699) using a forward primer (5'-CTGTAGAATTCGC-CATGAGCCACGGGAAGAGAAC-3') and a reverse primer (5'-ATAAGAAATGCGCGCCAGCTGGAGACGGCCATCAT-3'). The PCR product was digested with *Eco*RI and *Not*I followed by ligation into a *Eco*RI/*Not*I site of an expression vector, pcDNA3.1myc-His A (Invitrogen). The sequence of the subcloned insert was confirmed by sequencing of PCR products obtained using T7 forward and BGH reverse primers.

**Transfection of the Mouse Tis21 Gene into CHO-GD3-OAc<sup>-</sup> Cells**—Mouse Tis21-containing pcDNA 3.1 plasmid or empty vector was transfected into CHO-GD3-OAc<sup>-</sup> using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions, and the cells





**FIG. 1. Ganglioside profiles in CHO cells stably transfected with GD3 synthase (CHO-GD3 cells).** Flow cytometric analysis of CHO-GD3 cells is shown. Cells were stained with either mouse monoclonal antibody R24 (specific to GD3; upper panel) or 27A (specific to 9-O-acetyl-GD3; lower panel). Antibodies were precomplexed by anti-mouse IgG conjugated with phycoerythrin. Wild-type CHO cells showed no staining with either antibody (data not shown).

were incubated for 48 h. The cycle of transfection, 48-h incubation, and detachment from a culture plate was carried out three times, and re-expression of 9-O-acetyl-GD3 was monitored by flow cytometry using 27A.

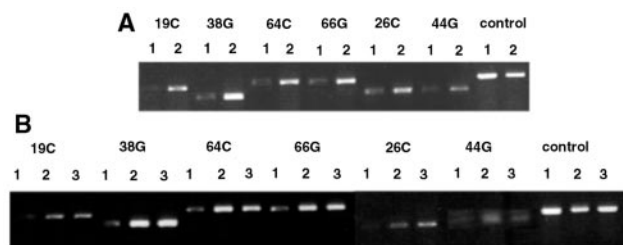
**Transfection of the ST6Gal-I Gene into Cells**—Human ST6Gal-I-containing pcDNA 3.1 plasmid or empty vector was transfected into wild-type, CHO-GD3, or CHO-GD3-OAc<sup>-</sup> mutant cells using LipofectAMINE Plus reagent (Invitrogen), and the cells were incubated for 48 h. Cells were detached by EDTA solution (without trypsin treatment) and subjected to flow cytometric analysis using CHE-FcD to detect 9-O-acetyl groups.

**Flow Cytometric Analysis**—Various types of CHO cells ( $1 \times 10^6$ ) were trypsinized to expose gangliosides (or released with EDTA only when studying glycoproteins) and washed with phosphate-buffered saline containing 1% bovine serum albumin. The cells were then incubated with either R24 antibody (for detecting GD3), 27A antibody (for detecting 9-O-acetyl-GD3), CD22-Fc (for detecting  $\alpha 2-6$ -linked sialic acids without 9-O-acetylation), or CHE-FcD (for detecting 9-O-acetyl sialic acids). The reagents were either precomplexed or complexed *in situ* with phycoerythrin-conjugated goat anti-mouse secondary antibody as previously described. After final washing, the fluorescent intensity on the cell surface was observed on a Becton Dickinson FACScan instrument.

## RESULTS AND DISCUSSION

**Isolation of a Clone of CHO-K1 Cells Stably Transfected with the GD3 Synthase Gene**—Wild type CHO cells express predominantly the monosialoganglioside GM3 (64–66). In our previous studies, we prepared CHO-K1 cells stably transfected with GD3 synthase (ST8Sia-I) (31). This cell population expressed not only disialoganglioside GD3 but also 9-O-acetyl-GD3 (detected with monoclonal antibodies R24 and 27A, respectively), neither of which are found in the wild type cells (31). During continuous culture, we noticed that this population of cells expressed varying levels of GD3 and 9-O-acetyl-GD3 (data not shown). We therefore used FACS to isolate clones with consistently high expression of GD3 and 9-O-acetyl-GD3 (see example in Fig. 1). As expected, stable clones expressing GD3 synthase (called CHO-GD3) expressed primarily GD3, as determined by a resorcinol stain of high performance thin-layer chromatography-separated total gangliosides (not shown), with smaller amounts of 9-O-acetyl-GD3 (also detected in the total lipid fraction of cells labeled with [<sup>3</sup>H]galactose and studied by autoradiography; data not shown).

**Isolation and Characterization of cDNAs Showing Enhanced Expression in CHO-GD3 Cells**—Although the CHO-GD3 cells



**FIG. 2. Comparison of expression levels of DD-PCR product genes.** A, RT-PCR of the six DD-PCR products predicted to show selective expression in CHO-GD3 cells (see Table I), studied in RNA from CHO-K1 wild type cells (lane 1) or the CHO-GD3 cells (lane 2). RT-PCR was performed using gene-specific primers for each product. B, RT-PCR for the six DD-PCR product genes was performed with the same gene-specific primer sets as in A, using RNA from CHO-K1 wild-type cells transiently transfected with the empty pcDNA 3.1 (lane 1) or transiently transfected with GD3 synthase in pcDNA 3.1 (lane 2) or another stable CHO-GD3 clone (lane 3).

express 9-O-acetyl-GD3, neither these cells nor the wild-type CHO-K1 cells express 9-O-acetyl GM3. Thus, a GD3-specific 9-O-acetyltransferase must either preexist in wild-type CHO-K1 cells or be secondarily induced by the presence of GD3. (Since GD3 synthase is a Golgi-localized protein with a short cytoplasmic tail, it is unlikely that such induction is a direct effect of the synthase protein itself.) To explore these possibilities as well as to evaluate the effects of GD3 and 9-O-acetyl-GD3 on the expression of other genes, we performed DD-PCR between RNA preparations from CHO-K1 wild type and CHO-GD3 cells using 80 arbitrary primers and three oligo(dT<sub>11</sub>N) (where N represents A, C, or G) primers. To minimize artifacts and false positive bands, DD-PCR using all primer pairs was repeated three times. Six DD-PCR products that consistently showed differentially enhanced expression in CHO-GD3 cells were finally obtained. These products were extracted, reamplified, subcloned, and sequenced. We designated them as 19C, 26C, 38G, 44G, 64C, and 66G, according to the numbering of the arbitrary primers and oligo(dT<sub>11</sub>N) reverse primer sets originally used for detection of each.

To confirm that message expression of these DD-PCR products was actually enhanced or induced in CHO-GD3, we performed RT-PCR using gene-specific primers for each product. As shown in Fig. 2A, 19C, 38G, 64C, and 66G were expressed in CHO-GD3 much more abundantly than in wild-type CHO-K1 cells. Expression of the other two products, 26C and 44G, was also increased in CHO-GD3 cells (Fig. 2A). These results confirm that expression of all six mRNAs is up-regulated by stable transfection of the GD3 synthase gene. Similar data were obtained by comparative RT-PCR between wild-type cells and another CHO-GD3 clone, as well as with wild-type cells transiently transfected with GD3 synthase (Fig. 2B). These results confirmed the consistent enhancement of expression of these genes by the presence of GD3.

**Nucleotide Sequence Analysis of DD-PCR Products Enhanced by Transfection of GD3 Synthase**—Nucleotide sequence analysis followed by BLAST homology searching of the NCBI data bases revealed similarities of each of the hamster DD-PCR products with other previously reported genes (see summary in Table I). Product 19C was most similar to the mouse Tis21/Btg2/PC3/APRO-1 gene (accession number M64292), which is a member of an anti-proliferative protein family (hereafter called the Tis21 gene) (67). The 64C sequence showed homology with mouse vascular cell adhesion molecule-1 (VCAM-1; accession number X67783) (68). The 38G sequence was found to be homologous to a small type II transmembrane protein of unknown function (accession number AB015632) (69). The 66G sequence is similar to a rat KC protein-like protein gene (ac-

TABLE I  
Genes similar to the DD-PCR products enhanced in CHO-GD3 cells in comparison with CHO-K1 wild type cells

DD-PCR product	Accession no.	Most homologous gene (percentage nucleotide identity)	Accession no.
19C	AB089669	Mouse <i>Tis21</i> gene (85%)	M64292
26C	AB089670	Rat FxC1 protein gene (93%)	AF061242
38G	AB089671	Human type II transmembrane protein gene (88%)	AB015632
44G	AB089672	Mouse SPR-1 gene (87%)	X91824
64C	AB089673	Mouse VCAM-1 gene (86%)	X67783
66G	AB089674	Rat KC protein gene (85%)	M86536

cession number M86536), which is known to be induced at early stages following cell stimulation by platelet-derived growth factor (70). The 26C sequence showed the most similarity to Rat FxC1 (accession number AF061242), a gene of unknown function, which is induced at a certain stage of a healing femur fracture (71). The 44G sequence is most similar to the rat SPR 1 protein gene (accession number X91824), a small proline-rich protein that is thought to be associated with cell proliferation but whose specific functions are unknown (72). Taken together, all of the above results indicate that transfection of GD3 synthase into CHO-K1 causes a major increase in expression of genes for VCAM-1, *Tis21*, the KC-protein-like protein, and a functionally unknown type II transmembrane protein and a small increase in expression of the FxC1 and SPR-1 genes. Combined with ganglioside profiles in the CHO-GD3 line, these data indicate that the six genes are specifically up-regulated by the expression of GD3 and/or 9-O-acetyl-GD3.

Liu *et al.* (73) showed that transfection of GD3 synthase gene into mouse neuroblastoma Neuro2a cells resulted in induction of several differentiation-associated genes. Notably, these genes are completely different from those that we have found to be up-regulated in CHO-GD3 cells. Thus, the genes secondarily induced by GD3 synthase are quite different between cell types and/or status (note that, unlike Neuro2a cells, CHO-K1 cells are devoid of any differentiation ability). The other possibility is that the difference of gene induction is due to an overall difference of ganglioside expression profile. Whereas CHO-GD3 cells express mainly GM3, GD3, and 9-O-acetyl-GD3, transfection of Neuro2a cells with the GD3 synthase gene induces not only GD3 but also other larger b-series gangliosides (74). Similar findings were observed in rat pheochromocytoma PC12 cells, in which transfection of the GD3 synthase gene resulted in drastic increase of GD1b and GT1b (75). Thus, the outcome of GD3 synthase expression could depend not only upon the cell type but also upon the preexisting ganglioside profile in the cells.

**Construction of 9-O-Acetyl-GD3-deficient but GD3-positive CHO Cells**—We also isolated mutant cells (called CHO-GD3-OAc<sup>-</sup>) that no longer express 9-O-acetyl-GD3 while continuing to express GD3. The cloned CHO-GD3 cells that showed consistently high expression of both GD3 (antibody R24-positive) and 9-O-acetyl-GD3 (antibody 27A-positive) were treated with the mutagen EMS at a dose that limited the mutation rate per cell to slightly above  $2 \times 10^{-4}$  (see “Experimental Procedures”). Cells were incubated with EMS and allowed to recover for 3 days. Subsequently, individual cells that had lost 9-O-acetyl-GD3 expression but continued to express GD3 were screened for by double color flow cytometric analysis using two antibodies, FITC-conjugated 27A and biotinylated R24. A clone with such properties was isolated by FACS. Compared with CHO-GD3 (Fig. 1A), these CHO-GD3-OAc<sup>-</sup> cells completely lacked immunoreactivity against antibody 27A, whereas R24 antibody staining remained unchanged (Fig. 3).

**Expression of the *Tis21* Gene Is Markedly Reduced in CHO-GD3-OAc<sup>-</sup> Cells**—To examine whether expression of any of the six DD-PCR product genes up-regulated by GD3 synthase ex-

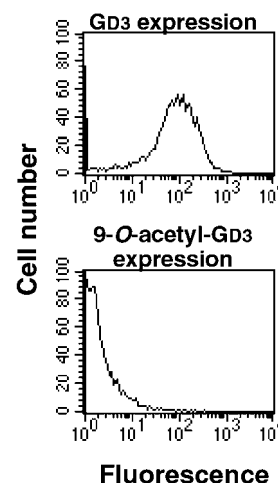


FIG. 3. Isolation of 9-O-acetyl-GD3-deficient CHO-GD3 mutant cells (CHO-GD3-OAc<sup>-</sup>). CHO-GD3 cells were treated with EMS and 9-O-acetyl-negative cells isolated by FACS as described under “Experimental Procedures.” Mutant cells were immunostained with either R24 (A) or 27A (B) monoclonal antibody precomplexed by the phycoerythrin-conjugated secondary antibody, as described in the legend to Fig. 1.

pression (Table I) was altered in the CHO-GD3-OAc<sup>-</sup> cells, we performed comparative RT-PCR of these mRNAs. As shown in Fig. 4, only the *Tis21* gene was found to show decreased expression in the mutant cells, whereas all of the others were detected at levels similar to those found in the parent line CHO-GD3. The unchanged expression levels of the other genes also confirm that they are enhanced by GD3 and not by 9-O-acetyl-GD3.

**Transfection of the *Tis21* Gene Restores 9-O-Acetyl-GD3 Expression in CHO-GD3-OAc<sup>-</sup> Cells**—There are three possible explanations for the above results. First, the reduction of *Tis21* gene expression may have led to the deficiency of 9-O-acetyl-GD3. Alternatively, the decrease of *Tis21* gene expression may have resulted from the deficiency of 9-O-acetyl-GD3 (*i.e.* *Tis21* gene expression was originally induced in CHO-GD3 cells by 9-O-acetyl-GD3). The third possibility is that the induction of *Tis21* and 9-O-acetylation are not causally related but rather are activated by a common upstream effector, which is up-regulated by GD3 expression. To differentiate between these possibilities, we transfected a cDNA for the *Tis21* gene into the CHO-GD3-OAc<sup>-</sup> cells and allowed the cells to recover for ~2 days. One round of transfection resulted in small but significant reexpression of 9-O-acetyl-GD3 after a 2-day incubation, in comparison with cells transfected with empty vector alone (data not shown). Since gangliosides may have a slow synthesis/turnover, we repeated the cycle of transient transfection and 2-day incubation. Flow cytometric analysis of the transfected cells after additional transfections showed increasing restoration of 9-O-acetyl-GD3 (see example after three rounds in Fig. 5). These results show that 9-O-acetylation of GD3 is up-regulated by the *Tis21* gene in a linear pathway and exclude

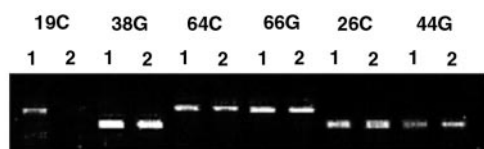


FIG. 4. Selective reduction of *Tis21* gene (19C) expression levels in CHO-GD3-OAc<sup>-</sup> cells. RT-PCR for the DD-PCR product genes using RNA obtained from CHO-GD3 (lane 1) or CHO-GD3-OAc<sup>-</sup> (lane 2) cells was performed using gene-specific primers for each DD-PCR product.

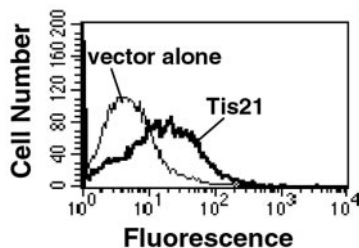


FIG. 5. Restoration of 9-O-acetyl-GD3 expression levels in CHO-GD3-OAc<sup>-</sup> cells by transient transfection of the *Tis21* gene. CHO-GD3-OAc<sup>-</sup> cells were immunostained with the 9-O-acetyl-GD3-specific antibody 27A followed by the phycoerythrin-conjugated secondary antibody and analyzed by flow cytometry after transfection of the empty vector (line) or a vector expressing *Tis21* (thick line). A progressive increase in 9-O-acetyl-GD3 expression levels was seen with each round of transfection. An example of the result from a third round of transfection is shown.

the possibility that expression of the *Tis21* gene was induced or enhanced by 9-O-acetyl-GD3. In addition, judging from the markedly reduced *Tis21* mRNA level, the mutation in the 9-O-acetyl-GD3 negative cells is likely to be in the promoter region of the *Tis21* gene or in further upstream gene(s) responsible for the induction of *Tis21* gene expression. Taken together, these results indicate that *Tis21* is up-regulated by GD3 and is then involved in the induction of 9-O-acetylation of GD3.

*9-O-Acetylation of Sialic Acid on N-Glycans Is Not Affected by Tis21*—Previously, we also showed that transfection of ST6Gal-I into wild-type CHO-K1 cells induced 9-O-acetylation exclusively on newly synthesized  $\alpha$ 2-6-linked sialic acids of endogenous N-linked glycans (31). To examine whether reduction of *Tis21* also abolished the potential to 9-O-acetylate  $\alpha$ 2-6-linked sialic acids, we transfected the ST6Gal-I gene into CHO-GD3-OAc<sup>-</sup> cells and investigated the generation of 9-O-acetyl sialic acid residues on glycoproteins by flow cytometry using a 9-O-acetyl sialic acid-specific probe, CHE-FcD, which is a soluble form of influenza virus C hemmagglutinin 9-O-acetyltransferase conjugated with a portion of the IgG1 Fc domain whose esterase activity was irreversibly inactivated by treatment with diisopropyl fluorophosphate (60, 76). As shown in Fig. 6, 9-O-acetyl sialic acids on proteins were detected by flow cytometric analysis of EDTA-released cells (Fig. 3), despite the continued absence of 9-O-acetylation of GD3 (detected by 27A after trypsinization; data not shown). Thus, the loss of *Tis21* gene expression is not functionally related to 9-O-acetylation of  $\alpha$ 2-6-linked sialic acids of N-glycans, supporting the notion that the mechanism of 9-O-acetylation of  $\alpha$ 2-8-linked sialic acids on gangliosides is different from that of  $\alpha$ 2-6-linked sialic acid on N-glycans. A different specific 9-O-acetyltransferase is either up-regulated by some other pathway that is turned on by the presence of the  $\alpha$ 2-6 linked sialic acid residues or is already present in the original CHO cells. Taken together, these data indicate that GD3 synthase expression induces the expression of the *Tis21* gene, which in turn, leads to the up-regulation of 9-O-acetyl-GD3 (Fig. 7). To our knowledge, this is

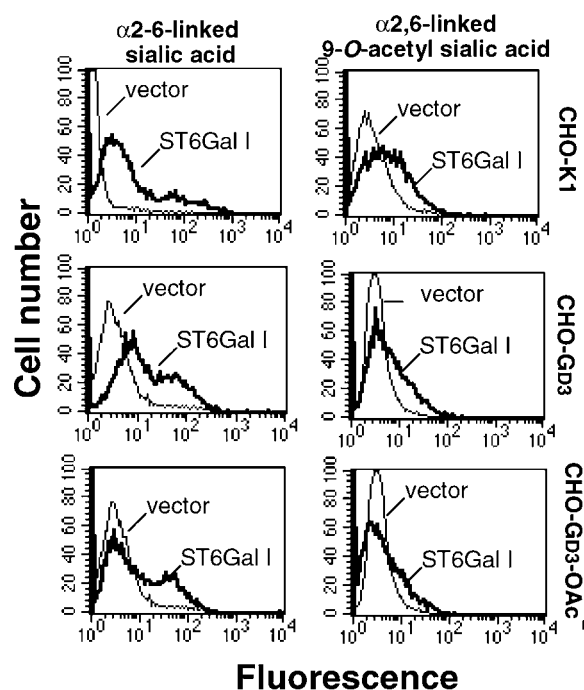


FIG. 6. Expression of  $\alpha$ 2-6-linked 9-O-acetyl sialic acids in CHO-GD3-OAc<sup>-</sup> cells transiently transfected with ST6Gal-I. Wild-type CHO-K1, CHO-GD3, or CHO-GD3-OAc<sup>-</sup> cells were transfected with pcDNA3.1-based ST6Gal-I and immunostained with CD22-Fc (to detect  $\alpha$ 2-6-linked sialic acid without 9-O-acetyl groups; left panels) or CHE-FcD (to detect 9-O-acetyl sialic acids; right panels), followed by the phycoerythrin-conjugated secondary antibody.

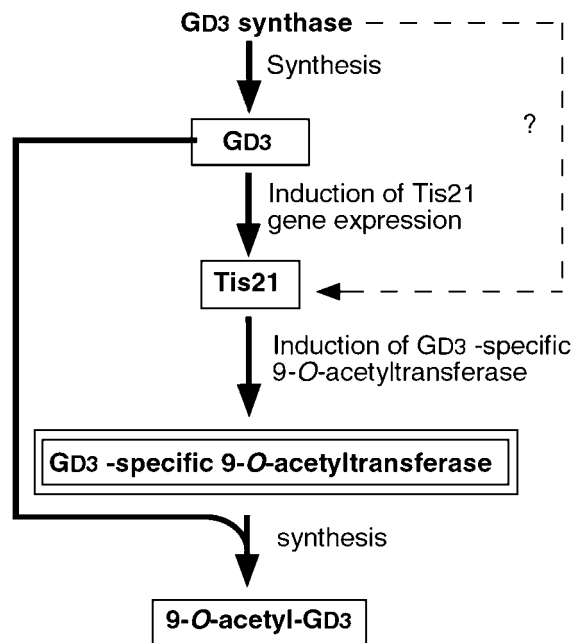


FIG. 7. Model for induction of 9-O-acetyl-GD3 by GD3 via the *Tis21* gene in CHO-K1 cells. The endogenously synthesized GD3 resulting from GD3 synthase expression enhances the expression of the *Tis21* gene, which in turn induces 9-O-acetylation of GD3 (presumably via induction of a 9-O-acetyltransferase specific for GD3). The unlikely but formal possibility that the GD3 synthase protein acts directly to induce *Tis21* expression is also indicated by the dotted line.

the first proposal of a mechanism for GD3-induced 9-O-acetylation of GD3.

The *Tis21* gene is a widely expressed but highly inducible member of an “anti-proliferative” protein gene family. This



soluble intracellular protein was first identified as one of the immediate early genes activated by nerve growth factor-treated PC12 cells and by 12-*O*-tetradecanoylphorbol-13-acetate ester-treated NIH3T3 cells (77, 78). *Tis21* was also shown to be up-regulated by the tumor suppressor gene p53 after DNA damage induced by genotoxic reagents (78–80) or during the embryogenesis of neurons when the cells differentiated or ceased to proliferate (67, 81, 82). Such findings have led to the suggestion that *Tis21* serves as an anti-proliferative factor through the regulation of cell cycle, causing cell arrest, which then allows either cell differentiation in response to nerve growth factor or cell repair in response to DNA damage. Indeed, stable transfection of the *Tis21* gene into NIH3T3 cells caused impaired transition from G<sub>1</sub> to S phase (81, 83). However, the anti-proliferative property is not consistent in all cases. Whereas *Tis21* expression is induced by nerve growth factor, fetal growth factor, interleukin-6, 12-*O*-tetradecanoylphorbol-13-acetate, serum, and epidermal growth factor, the first three stimuli induce neuronal differentiation, whereas the latter stimuli induce proliferation (67, 80). It has been suggested that response of a particular cell depends on the duration of activation of the Ras/MEK/mitogen-activated protein kinase pathway (80).

As mentioned above, *Tis21* is also a marker of neurogenesis. It is transiently expressed in neuroepithelial cells in the ventricular zone preceding neural differentiation into mature neurons (82). Interestingly, this is also the originally described distribution of 9-*O*-acetyl GD3 in the developing nervous system (84). GD3 itself is also expressed embryonically in neuronal cells, and the level of GD3 decreases during neural differentiation (85). Furthermore, the involvement of 9-*O*-acetyl-GD3 in differentiation, development, and migration of some central neuronal cells has been suggested (46, 86–89). These findings imply that 9-*O*-acetyl GD3 and GD3 are functionally linked to the *Tis21* gene at least in some tissues. However, the generality of induction of 9-*O*-acetylation via *Tis21* gene up-regulation by GD3 synthesis and the functional correlates of this process remain to be explored. Since 9-*O*-acetyl-GD3 is a well known tumor-associated antigen of human melanoma (17, 90–92) and basal cell carcinoma (20, 93), studies of *Tis21* in these tumors are also warranted.

One of our ultimate goals in these studies is to isolate and characterize the specific 9-*O*-acetyltransferase involved in generating 9-*O*-acetyl-GD3. Since many biochemical studies indicate that such 9-*O*-acetyltransferases are localized in the Golgi apparatus (26, 27, 29, 30), *Tis21* (a cytosolic protein involved in suppression of cell proliferation) (78, 79) is not a likely candidate to be a physical part of 9-*O*-acetyltransferase itself. Others have also attempted to isolate the 9-*O*-acetyltransferase without success. In one study, a putative acetyl-CoA transporter that induced 9-*O*-acetylation of GD3 was isolated through expression cloning (36). However, this protein has an ortholog in yeasts (which do not express sialic acids) and is predominantly expressed in the endoplasmic reticulum. Another group attempted expression cloning of this 9-*O*-acetyltransferase but instead isolated a secreted mucin-like molecule similar to the milk fat globule protein (35). Our similar attempts at expression cloning only yielded a contaminating bacterial transcriptional repressor gene that induced 9-*O*-acetylation of GD3 in COS cells transfected with GD3 synthase (37). Similar problems have plagued attempts to expression-clone the 9-*O*-acetyltransferases involved in 9-*O*-acetylation of glycoproteins, yielding candidates such as a truncated form of a vitamin D-binding protein (37), which again could not be a component of the Golgi 9-*O*-acetyltransferase. All of these studies were also confounded by the fact that GD3 synthase-expressing COS cells

already have a low level of 9-*O*-acetyl-GD3.<sup>2</sup> Recently, Huang *et al.* (94) showed that 9-*O*-acetylation of GD3 was up-regulated by interleukin-4 and -13 and down-regulated by interferon- $\gamma$  in basal layer keratinocytes in chronic lesions. Such findings further indicate that the synthesis of 9-*O*-acetyl-GD3, namely the expression and/or activation of GD3 9-*O*-acetyltransferase, is regulated by multiple endogenous and exogenous factors. This, together with the possibility that the 9-*O*-acetyltransferase may consist of an unstable complex of multiple subunits, may explain the many failures to characterize the 9-*O*-acetyltransferase by a variety of biochemical and molecular approaches.

We also attempted to determine whether any of the same genes are up-regulated in cells transfected with ST6Gal-I, in which selective 9-*O*-acetylation of *N*-glycans is seen. However, we have been unable to generate the cell line required to do these studies. Whereas the primary data in this paper are based on a stable clone overexpressing GD3, it has been not been possible for us (for unknown reasons) to obtain a stable ST6Gal-I-expressing clone despite many attempts; in particular, the level of ST6Gal-I expression and 9-*O*-acetylation of *N*-glycans varies and disappears with time in culture (data not shown). On the other hand, we felt that direct comparisons among transiently transfected cells would not be valid, since the efficiency of transient transfection cannot be controlled, and unrelated genes are likely to be temporarily up-regulated during such a process. We are, however, able to say that *Tis21* up-regulation was not detected by PCR studies of CHO cells transiently transfected with ST6Gal-I,<sup>3</sup> further indicating that *Tis21* induction by GD3 synthase is indeed a very specific process.

In our present study, we employed DD-PCR and mutagenesis analysis using some cell variants that originated from the single parental cells (*i.e.* wild type CHO-K1, GD3- and 9-*O*-acetyl-GD3-stably expressing CHO-cells (CHO-GD3), and 9-*O*-acetyl-GD3-deficient CHO-GD3 mutant (CHO-GD3-OAc<sup>-</sup>)). This approach was expected to minimize the possibility of characterizing biologically unrelated factors for 9-*O*-acetylation of GD3 even if they exhibited induction of 9-*O*-acetylation. There are multiple possible reasons for the lack of detection of the GD3-9-*O*-acetyltransferase gene itself, such as low level expression of mRNA encoding for this Golgi enzyme or masking of the 9-*O*-acetyltransferase gene DD-PCR product by other abundantly expressed gene(s). Regardless, the DD-PCR between CHO-K1 wild type and CHO-GD3 cells showed that six genes are specifically turned on by the presence of GD3 on CHO cells, and the present data do demonstrate that GD3-induced 9-*O*-acetylation is mediated via the *Tis21* gene in CHO-K1 cell lines (Fig. 7). Evidently, the endogenously synthesized GD3 resulting from GD3 synthase expression somehow enhances the expression of the *Tis21* gene, and this in turn induces 9-*O*-acetylation of GD3, presumably via induction of a 9-*O*-acetyltransferase that is specific for GD3. Of course, we cannot rule out the possibility that the GD3 synthase protein acts directly in some unknown way to induce *Tis21* expression (Fig. 7). However, as stated earlier, this seems unlikely, since this enzyme is primarily confined to the lumen of the Golgi apparatus and has a short cytoplasmic tail.

Definition of this pathway also provides useful new tools for the characterization of GD3-9-*O*-acetyltransferase. For example, comparison of gene expression profiles between CHO-GD3-OAc<sup>-</sup> cells and those stably or transiently transfected with *Tis21* by DD-PCR or another method such as gene subtraction could be a more promising approach. We also need to study some of the

<sup>2</sup> H. Satake and A. Varki, unpublished observations.

<sup>3</sup> H. Satake, H. Y. Chen, and A. Varki, unpublished observations.

other DD-PCR products, such as the type 2 membrane protein of unknown function. Such studies are in progress.

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