Expression Cloning and Functional Characterization of Human cDNA for Ganglioside G<sub>M3</sub> Synthase*

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Ganglioside G<sub>M3</sub> is a major glycosphingolipid in the plasma membrane and is widely distributed in vertebrates. We describe here the isolation of a human cDNA whose protein product is responsible for the synthesis of G<sub>M3</sub>. The cloned cDNA spanned 2,359 base pairs, with an open reading frame encoding a protein of 362 amino acids with a predicted molecular mass of 41.7 kDa. The deduced primary structure shows features characteristic of the sialyltransferase family, including a type II transmembrane topology and the sialylmotifs L at the center and S at the C-terminal region. An amino acid substitution from aspartic acid to histidine was demonstrated at a position invariant in sialylmotif L of all the other sialyltransferases so far cloned. The best acceptor substrate for the gene product was lactosylceramide, and cells transfected with the cloned cDNA clearly exhibited de novo synthesis of G<sub>M3</sub>, with a measurable decrease in the precursor lactosylceramide. Despite the ubiquitous distribution of ganglioside G<sub>M3</sub> in human tissues, a major 2.4-kilobase transcript of the gene was found in a tissue-specific manner, with predominant expression in brain, skeletal muscle, and testis, and very low expression in liver.

It is known that sialic acid-containing glycosphingolipids, gangliosides, have various important biological functions (1, 2), and their functions as well as their biosynthesis are currently clarified. In vertebrates, almost all the ganglio-series gangliosides are synthesized from a common precursor, ganglioside G<sub>M1</sub>, which has the simplest structure among the major gangliosides. G<sub>M3</sub> itself is known to participate in induction of differentiation (3, 4), modulation of proliferation (2, 5), maintenance of fibroblast morphology (6), signal transduction (7), and integrin-mediated cell adhesion (8).

Molecular cloning of genes whose protein products catalyze the transfer of sialic acid through an α-2,3 linkage has been reported (14–17), and these protein products had the same acceptor substrates, i.e., the oligosaccharides of O- and N-linked glycoproteins and glycolipids. However, none of the gene products so far reported is known to be involved in the synthesis of ganglioside G<sub>M3</sub>. We demonstrated previously that the level of G<sub>M3</sub> synthase activity was dramatically enhanced during the monocytic differentiation of HL-60 cells (21). Using a cDNA library prepared from the differentiated HL-60 cells, we isolated a cDNA encoding the G<sub>M3</sub> synthase by a modified expression cloning method. In the present study, we demonstrate that the G<sub>M3</sub> synthase shows some features clearly distinct from those of all the other sialyltransferases so far cloned, although it possesses several features common to members of the sialyltransferase family.

MATERIALS AND METHODS

Cell Culture—The human myelogenous leukemia cell line HL-60, and the mouse lung carcinoma cell line 3LL-J5 (22), which completely lacks acidic glycosphingolipids, and its derivative cell lines, 3LL-HK46, which stably expresses the polyloma virus large tumor antigen, and 3LL-ST28, which is a line permanently transfected with human G<sub>M3</sub> synthase cDNA, were cultured in RPMI 1640 medium containing 10% fetal calf serum. HL-60 cells were inoculated at 2–3 × 10<sup>5</sup> cells/ml and treated with 24 nM TPA for 48 h, and pol(A)<sup>+</sup> RNA was isolated using a Fast Track mRNA isolation kit (Invitrogen).

Construction and Screening of cDNA Library—After reverse transcription, followed by double-strand synthesis and blunt-ending of the termini, cDNA was ligated with BosXI adapter (Invitrogen) and cloned into pCEV18, which is a derivative of pCEV7 (24). The cDNA library was divided into eight parts, and each part was amplified by PCR. The amplified DNA was digested with SmaI (Toyobo Co. Ltd.), and ligated to the pBKCMVh2,8ST vector. The vector was transformed into DH10B (Life Technologies, Inc.). After purification with Qiagen Tip (Qiagen), 100 μg of the plasmid DNA was introduced into 5 × 10<sup>5</sup> 3LL-HK46 cells by electroporation (180 V, 600 μF). Thirty-six to 48 h after transfection, the cells were harvested, washed with PBS(−), and reacted with anti-G<sub>M3</sub> mAb M2590 for 30 min on ice and stained with FITC-conjugated rabbit anti-mouse IgG/A/M (Zymed Laboratories Inc.) for 30 min on ice. The immunologically stained cells were isolated using an EPICS Elite ESP cell sorter (Coulter), and the plasmid DNA was rescued from the 5% of the cells sorted into the strongest fluorescent fields. After two rounds of the transfection and the sorting, 5 × 10<sup>5</sup> 3LL-HK46 cells were co-transfected with both 100 μg of the plasmid DNA rescued from the strongly fluorescent cells and 20 μg of pBKCMVh2,8ST, harboring human G<sub>M3</sub> synthase cDNA (19), and G<sub>M3</sub> expression on the host cell surface was detected using anti-G<sub>M3</sub> mAb R24 as a specific marker for enrichment of the targeted cDNA. The 0.6% of the total cells applied that were distributed into the strongest fluorescent fields were sorted using a FACS Vantage cell sorter (Becton

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1 The abbreviations used are: G<sub>M3</sub>, NeuAcα2–3Galβ1–4Glcβ1–1 Cer; G<sub>M3</sub>, GalNAcβ1–4NeuAcα2–3Galβ1–4Glcβ1–1 Cer; G<sub>M3</sub>, Galβ1–3GalNAcβ1–4 (NeuAcα2–3Galβ1–4Glcβ1–1 Cer (ganglioside nomenclature is based on that of Svennerholm (35)); TPA, 12-O-tetradecanoylphorbol-13-acetate; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; bp, base pairs; PBS, phosphate-buffered saline.
DNA was co-transfected into the host cells with the plasmid rescued by the method of Hirt (25). Thus enriched plasmid to cell sorting. The 5% of the cells sorted into the strongest into the host cells by electroporation. For the first two cycles of the monocytic lineage with phorbol ester and was introduced gene probe to control for the amount and quality of RNA in each sample.

Northern Blot Analysis—MTN blots (CLONTECH) were used to determine the tissue distribution of Gm3 synthase mRNA. A 2,068-bp EcoRI-EcoRI fragment of the pCEV4C7 cDNA was gel-purified, labeled with [α-32P]dCTP, and used as a probe. The blots were also hybridized with a radiolabeled human glyceraldehyde-3-phosphate dehydrogenase gene probe to control for the amount and quality of RNA in each sample.

RESULTS

Cloning of a Candidate cDNA for Gm3 Synthase—We first found that newly synthesized ganglioside Gm3 was barely detectable on the surface of recipient 3LL cells using anti-Gm3 monoclonal antibody M2590. We therefore devised a strategy for cloning the Gm3 synthase gene on the basis of the facts that anti-Gm3 monoclonal antibody R24 is an IgG protein, whereas M2590 mAb is an IgM protein, and that the Gm3 synthetic sialyltransferase (sialyltransferase-II, SAT-II) cDNA had been successfully cloned and was available for our use (21); the polyclonal virus large T antigen was first introduced into the mouse lung carcinoma cell line 3LL-J5 (22) to produce the 3LL-HK46 cell line, both of which were completely deficient in gangliosides but rich in lactosylceramide (the precursor of ganglioside Gm3). Thereafter, transfecting human Gm3 synthase cDNA into 3LL-HK46 cells, we successfully established the 3LL-ST28 cell line, which stably expressed ganglioside Gm3 synthase activity.

3LL-HK46 cells were used as host cells for the first stage of our expression cloning, in which co-transfection was performed with Gm3 synthase cDNA to detect the Gm3 expression on the cell surface. Then, in the second stage, 3LL-ST28 cells served as recipient cells to efficiently isolate the Gm3 synthase gene. The cDNA library was prepared in the vector pCEV18 from poly(A)+ RNA of HL-60 cells that had been differentiated along the monocytic lineage with phorbol ester and was introduced into the host cells by electroporation. For the first two cycles of cell sorting, the transfected cells were stained with M2590 followed by a FITC-conjugated second antibody and subjected to cell sorting. The 5% of the cells sorted into the strongest fluorescent fields was recovered, and the plasmid DNA was rescued by the method of Hirt (25). Thus enriched plasmid DNA was co-transfected into the host cells with the plasmid pBKCMVh2.SST (19), which harbors human Gm3 synthase cDNA. After sorting the cells that were stained with anti-Gm3 monoclonal antibody R24 and FITC-conjugated anti-mouse IgG antibody, we obtained a single candidate clone (termed 4C7) via two sequential sibling selections using 3LL-ST28 cells as the hosts. Fig. 1 shows the FACS profile of 3LL cells transiently transfected with either 100 μg of pCEV18 (A, C) or pCEV4C7 (B, D). Forty-two hours thereafter, the cells were reacted either with anti-Gm3 monoclonal antibody R24 (A, B) or with anti-Gm3 monoclonal antibody M2590 (C, D).

**Fig. 1.** Flow cytometric analyses of ganglioside Gm3 expression in mouse lung carcinoma cells. 3LL-ST28 (A, B) and 3LL-HK46 (C, D) cells were transiently transfected with either 100 μg of pCEV18 (A, C) or pCEV4C7 (B, D). Forty-two hours thereafter, the cells were reacted either with anti-Gm3 monoclonal antibody R24 (A, B) or with anti-Gm3 monoclonal antibody M2590 (C, D).
acceptor. It has not yet been determined whether the 4C7 product is active toward free lactose or N- and O-linked oligosaccharides of glycoproteins as substrates. However, this strict acceptor specificity for lactosylceramide, and not for the non-reducing terminal galactose moieties of other glycosphingolipids tested, together with previous reports on the substrate specificity of purified GM3 synthase, suggest the existence of isozymes having broader substrate specificity than the 4C7 gene product. The glycosphingolipid composition in clone 4C7-transfected 3LL-HK46 cells was analyzed by TLC, and it was clearly demonstrated that the de novo synthesis of GM3 molecules occurred in the transfected cells, along with an observable decrease in neutral lactosylceramide. The hydropathy plot (Fig. 2B) showed three or four strongly hydrophobic segments in and around sialylmotif S near the C terminus of the enzyme. These results suggest that the enzyme might be anchored to the luminal side of the Golgi membrane.

**Northern Blot Analysis of Human GM3 Synthase Transcripts**—The expression of GM3 synthase in human tissues was assessed by Northern blot analyses. A major transcript 2.4 kilobases in size was detected at various level in all human tissues tested; it was highly expressed in brain, placenta, skeletal muscle, and testis, whereas it was very weakly expressed in liver, kidney, pancreas, and colon. In some tissues, a minor band of 7 kilobases was detected (Fig. 4A). To characterize in more detail the gene expression of GM3 synthase in brain, Northern blotting analysis of various parts of the human brain was performed with the same probe. As shown in Fig. 4B, GM3 synthase mRNA was widely distributed in human brain, but slightly elevated expression was observed in the cerebral cortex, temporal lobe, and putamen.

**DISCUSSION**

This study describes the molecular cloning of a new human sialyltransferase involved in the biosynthesis of ganglioside GM3. In our expression cloning, it was found that newly synthesized ganglioside GM3 was barely detectable with anti-GM3 antibody M2590 on the surface of transfected 3LL-HK46 cells in FACS analysis (Fig. 1). This problem was overcome by co-transfecting or introducing in advance the GD3 synthase cDNA; the final product ganglioside GD3 was easily detected using its specific antibody R24 (Fig. 1). Such modifications of expression

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**Fig. 2**—continued

**A** The nucleotide and its deduced amino acid sequences of clone 4C7. The nucleotide sequence is numbered on the left, with sequences upstream of the putative initiating ATG assigned negative numbers. The predicted transmembrane domain is underlined and the potential N-glycosylation sites are marked with triple dots below the Asn residues. Sialylmotifs L and S are double-underlined, and the characteristic His residue in the sialylmotif L is boxed. A, the sequence of clone 4C7. B, hydropathy plot of the 4C7 product analyzed by the method of Hopp and Woods (30). The solid bars below the plot indicate the locations of sialylmotifs L and S, comparison of amino acid sequence of sialyltransferase of human sialyltransferase so far cloned. SAT-I, clone 4C7 product; ST3N-1, Galβ1–3(4)GlcNAcα2,3-ST (15); ST3N-2, Galβ1–3GalNAcα2,3-ST-1 (14); STO-1, Galβ1–3GalNAcα2,6-ST (16); ST3O-1, Galβ1–3GalNAcα2,6-ST (17); SThM, sthm gene (GenBank data base, accession number U14550); ST6N, Galβ1–4GlcNAcα2,6-ST (18); SAT-II, GM3 synthase (19, 20); STX, sfx gene (12); ST8SIalII, Sialα2–3Galβ1–4GlcNAcα2,8-ST (GenBank data base, accession number AF004668);
cloning are generally useful, and use of an analogous modification has also been reported (20).

The human G\textsubscript{M\textsubscript{3}} synthase cDNA cloned in the present study and its product showed some characteristics strikingly different from those of the other sialyltransferases so far cloned. An invariant aspartic acid residue was found in the C-terminal region of the sialylmotif L of all the other sialyltransferases (Fig. 2C). This acidic amino acid residue was substituted by the basic amino acid (His) in G\textsubscript{M\textsubscript{3}} synthetic sialyltransferase. More interestingly, this amino acid replacement is conserved in the G\textsubscript{M\textsubscript{3}} synthases of mouse, rat, and green monkey origins. Thus, this replacement must be critically relevant to the G\textsubscript{M\textsubscript{3}} synthetic activity. It is, however, still unclear how this substitution affects the interaction between the enzyme and its substrate(s) and whether or not the substitution is limited to G\textsubscript{M\textsubscript{3}} synthases of mammalian origin.

Among all the glycosphingolipids tested, only lactosylceramide could serve as an acceptor for the sialylation catalyzed by the cloned human G\textsubscript{M\textsubscript{3}} synthase. In contrast, it has been reported that the purified G\textsubscript{M\textsubscript{3}} synthase from rat liver exhibited a broader substrate specificity (27) and that the enzyme from rat brain could utilize both galactosylceramide and asialoganglioside G\textsubscript{M\textsubscript{3}} (G\textsubscript{A\textsubscript{2}}) as acceptors as well as lactosylceramide (31). Although we cannot rule out differences in the purity of the enzyme preparations, these discrepancies may imply the existence of isozymes with different substrate specificities for glycosphingolipids.

Northern analysis indicated that the human G\textsubscript{M\textsubscript{3}} synthase gene was expressed in a tissue-specific manner (Fig. 44). The high expression of G\textsubscript{M\textsubscript{3}} synthase messenger RNA in brain (Fig. 4B) may be responsible for the abundance in brain of G\textsubscript{M\textsubscript{3}} itself and of other gangliosides with more complex oligosaccharide chains, which might be related to axonal (32, 33) and dendritic outgrowth (34). The expression of G\textsubscript{M\textsubscript{3}} synthase was enhanced by the treatment of HL-60 and U937 cells with the differentiation inducer TPA (4). Although transcriptional regulation of G\textsubscript{M\textsubscript{3}} synthase has not yet been demonstrated, elucidation of the mechanisms controlling expression of the G\textsubscript{M\textsubscript{3}} synthase gene should yield insight into the mechanism(s) by which leukemic cells can be induced to differentiate into monocytoids.

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