Expression Cloning of Mouse cDNA of CMP-NeuAc:Lactosylceramide α2,3-Sialyltransferase, an Enzyme That Initiates the Synthesis of Gangliosides*

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Expression cloning of a cDNA for the α2,3-sialyltransferase (GM3 synthase) (EC 2.4.99.-) gene was performed using a GM3-lacking mouse fibroblast line L cell and anti-GM3 monoclonal antibody. Plasmids from a cDNA library generated with poly(A)+ RNA of a mouse fibrosarcoma line CMS55 and pdl3027 (polyoma T antigen) were co-transfected into L cells. The isolated cDNA clone pM3T-7 predicted a type II membrane protein with 13 amino acids of cytoplasmic domain, 17 amino acids of transmembrane region, and a large catalytic domain with 329 amino acids. Introduction of the cDNA clone into L cells resulted in the neo-synthesis of GM3 and high activity of α2,3-sialyltransferase. Among glycosphingolipids, only lactosylceramide showed significant activity as an acceptor, indicating that this gene product is a sialyltransferase specific for the synthesis of GM3. An amino acid sequence deduced from the cloned cDNA showed the typical sialyl motif with common features among α2,3-sialyltransferases. Among various mouse tissues, brain, liver, and testis showed relatively high expression of a 2.3-kilobase mRNA, whereas all tissues, more or less, expressed this gene.

Gangliosides are acidic, sialic acid-containing glycosphingolipids and are ubiquitously expressed in all tissues and cells (1, 2). Their carbohydrate structures are strictly regulated, depending on the development of tissues, differentiation, and malignant transformation of cells (3, 4). Ganglioside synthesis is catalyzed by glycosyltransferases, and the combination of expression levels of various glycosyltransferase genes determines characteristic patterns of ganglioside expression (5–8). Among the gangliosides, GM3 is the simplest, having only three sugars, and is widely distributed in all over the body of vertebrates (1). Because GM3 is a precursor for all other ganglioside series gangliosides and hematiosides (7), its biosynthesis strongly affects the levels of the more complex gangliosides.

Recent progress in the molecular cloning of glycosyltransferase genes responsible for the synthesis of gangliosides has enabled us and other investigators to analyze the regulatory mechanisms for the ganglioside expression and biological functions of them by manipulation of those genes to modify the ganglioside profiles in cultured cells and in experimental animals (9). In our laboratory, we have isolated genes encoding ganglioside GM2/GD2 synthase (10), GD3 synthase gene (11), and GM1/GD1b/GA1 synthase (12) and have analyzed expression of these genes in various tissues and malignant cells (8, 13, 14). We have also established gene knock-out mice lines in which all complex ganglioside series gangliosides are genetically eliminated in the whole body (15). The resulting phenotypic changes in those mutant mice pose new questions about the constitutive roles of simpler ganglioside structures (e.g. GM3 and GD3) for the eliminated complex gangliosides.

In the present study, we isolated a mouse cDNA clone that determines expression of ganglioside GM3 and have analyzed its expression pattern in various tissues. This gene product corresponds to GM3-specific sialyltransferase (CMP-NeuAc: lactosylceramide α2,3-sialyltransferase, α2,3S-T) (EC 2.4.99.-). Because of the critical role that GM3 synthase plays in the biosynthesis of all gangliosides, this probe will be extremely useful for the analysis of the biological functions of acidic glycosphingolipids.

MATERIALS AND METHODS

Tissue Cultures and Cells—Cell lines were cultured in Dulbecco’s modified Eagle’s minimal essential medium supplemented by 10% fetal bovine serum. L cell and B78 (a subline of mouse B16 melanoma) were kindly provided by Dr. A. P. Albino at Sloan-Kettering Cancer Center in New York. L cell is a mouse fibroblast line with no α2,3-sialyltransferase activity (for lactosylceramide acceptor) (16) and minimal GM3 expression. B78 expresses GM3 ganglioside almost exclusively (10).

Expression Cloning of Mouse GM3 Synthase cDNA—Plasmids of the cDNA library of mouse fibrosarcoma CMS55 was prepared using poly(A)+ RNA and the pCDM8 expression vector (Invitrogen, San Diego, CA). This library contained 5.0 × 10⁶ independent colonies. The strain of bacterial host used was Escherichia coli MC1061/P3.

1 The abbreviations used are: GM2, GalNAcβ1→4(NeuAcα2→3Galβ1→4Glc Cer; GD2, GalNAcβ1→4(NeuAcα2→3Galβ1→4Glc Cer; GD3, NeuAcα2→3 NeuAcα2→8 NeuAcα2→8 NeuAcα2→2,3Galβ1→4Glc Cer; GM3, NeuAcα2→3Galβ1→4Glc Cer; Lac-Cer (CDH), Galβ1→4Glc Cer; GA2, GalNAcβ1→4Glc Cer; Galβ1→4Glc Cer; GM1, Galβ1→4GlcNacβ1→4Galβ1→4Glc Cer; GA2, GalNAcβ1→4Glc Cer; Galβ1→4Glc Cer; GA1, Galβ1→4GlcNacβ1→4Galβ1→4Glc Cer; GD1a, NeuAcα2→3 Galβ1→4Glc Cer; GA1, Galβ1→4GlcNacβ1→4Galβ1→4Glc Cer; GA2, NeuAcα2→3Galβ1→4Glc Cer; GD1b, NeuAcα2→3Galβ1→4GlcNacβ1→4Galβ1→4Glc Cer; GD2a, NeuAcα2→8 NeuAcα2→8 NeuAcα2→2,3 Galβ1→4Glc Cer; GD2b, NeuAcα2→8 NeuAcα2→2,3 Galβ1→4Glc Cer (ganglioside nomenclature is based on that of Svennerholm (47); α2,3S-T, α2,3-sialyltransferase (the nomenclature of sialyltransferases is based on that of Tsuji et al. (48)).

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9271
Expression Cloning of GM3 Synthase cDNA

(Conring, Corning, NY), were co-transfected with 8 µg each of cDNA library plasmid and pdl3027. After 48–60 h, the transfected cells were detached from plates and incubated with a mouse monoclonal antibody M2590 (mouse IgM) (18) (Nihon Biotest Research Institute, Tokyo, Japan) at 20 µg/ml on ice for 45 min. After washing, cells were plated on dishes coated with goat anti-mouse IgM (Cappel, Durham, NC) as described previously (10). Plasmid DNA was rescued from the transfected pools by preparing Hirt extracts and transformed into MC1061/F3. Expanded plasmids were transfected again, and the same procedure was repeated five times. Using isolated colony extracts and microscale DEAE-dextran transfection and immunofluorescence assay, we isolated a cDNA clone that determines the expression of ganglioside GM3 in L cells.

DNA Sequencing—The sequence was determined by dyeoxynucleotide terminal sequencing using the PRISM dye terminator cycle sequencing kit and model 373 DNA sequencer (Applied Biosystems, Foster City, CA). The cDNA insert of the cloned pM3T-7 was cleaved by Hind III and Not I and then inserted into Hind III/Not I sites of phagemid BlueScript® vector. Deletion mutants of this clone were prepared with a Kilo-Sequence deletion kit (Takara, Kyoto).

Transfection of the Cloned cDNA—Isolated cDNA clone pM3T-7 (in pCDM8) was transfected into L cells by the DEAE-dextran method, and the transfected cells were used for the analyses of ganglioside expression and of the enzyme activity after 60 h. Plasmid pM3T-7/MIKneo was constructed by inserting XhoI/Not I fragment of pM3T-7 into XhoI/Not I sites of pMIKneo expression vector (kindly provided by Dr. K. Maruyama at Tokyo Medical Dental School).

Enzyme Assay—The enzyme activity of α2,3S-T was measured as described previously (19). Briefly, to prepare membrane fractions, samples were lysed using a nitrogen cavitation apparatus. Nuclei were removed by low speed centrifugation, and the supernatant was centrifuged at 105,000 g for 20 min. The enzyme activity was evaluated by using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan) at 20°C. Nuclei of the enzyme after 60 h. Plasmid pM3T-7/MIKneo was constructed by inserting XhoI/Not I fragment of pM3T-7 into XhoI/Not I sites of pMIKneo expression vector (kindly provided by Dr. K. Maruyama at Tokyo Medical Dental School).

Results

Isolation of α2,3S-T cDNA Clones—L cells were transfected with a cDNA library prepared from CMS5j and pdl3027 containing the polyoma T gene. After five cycles of transfection, panning, and Hirt extraction, a cDNA clone pM3T-7 was obtained.

Synthesis of GM3 in the Transfectant Cells of the Cloned cDNA—pM3T-7 in pCDM8 or pMIKneo vector was transiently introduced into L cells, which lack GM3 expression and GM3 synthase activity (16), to confirm in flow cytometry whether the clone determines the expression of GM3 on the cell surface. As shown in Fig. 1, L cells transfected with pM3T-7 expressed definite amount of GM3, whereas those transfected with the vector alone did not. Glycosphingolipids extracted from the transfected cells were analyzed to confirm GM3 synthesis. As shown in Fig. 2, the transfectant cells with pM3T-7/MIKneo showed a definite GM3 band in TLC, although the parent cell (data not shown) and the transfected cells with pMIKneo alone showed a very faint band, suggesting that the parent L cell expresses a minimal level of the enzyme.

Sequence of the Insert of pM3T-7—Fig. 3 shows the entire sequence of the insert of pM3T-7 determined by sequence analysis of the original pM3T-7 in pCDM8 or deletion constructs of phagemid BlueScript® containing XhoI/Not I fragment. Total size of this insert was 1710 base pairs comprising a 12-base pair 5′-untranslated region, a continuous open reading frame of 1077 base pairs, and 621 base pairs of 3′-untranslated region. The initiation codon at the beginning of the open reading frame is embedded within a sequence similar to the Kozak consensus initiation sequence (23, 24). This open reading frame predicts a 359-amino acid protein with a molecular mass of 41,244 daltons. Search of currently available protein and nucleic acid databases identified a number of genes with significant sequence homology to this cDNA. A majority of those genes were sialyltransferases, and highly homologous regions were detected at the sequences of sialylmotif L, sialyl motif S, and a few other regions. Among sialyltransferase cDNAs, ST3Gal III (ST3N) (Galβ1,3/1,4GlcNAc α2,3S-T) generally
Expression Cloning of GM3 Synthase cDNA

We have identified a cDNA that determines the expression of GM3 and induces α2,3S-T activity in transfected cells. Furthermore, the deduced amino acid sequence indicated the presence of the sialyl motifs, which has been recognized among all mammalian and chicken sialyltransferases cloned so far (26), suggesting that this cDNA represents the GM3 synthase gene. Examination of the sequence showed features in common with other α2,3-sialyltransferases. As shown in Fig. 6, the derived GM3 synthase sequence contained a number of characteristic regions commonly detected only in the sequences of ST3Gal α2,3-sialyltransferases (see enclosed boxes in sialyl motif L and S). These regions may be involved in the recognition of acceptor structures. The conserved YPE sequence was also noted by Sasaki in a recent review (27). In addition, the ST8Sia group showed a common sequence (NPS) in the carboxyl terminus region of the sialyl motif L. This sequence contrasted with the sequence YPE, which was commonly detected among ST3Gal group, supporting the idea that this region might be involved in the acceptor recognition.

The properties of the GM3 synthase predicted from the amino acid sequence (Fig. 3, A and B) and demonstrated by the analysis with extracts from cDNA-transfected cells were not so different from those of previously purified GM3 synthases. For example, $K_m$ for lactosylceramide and CMP-NeuAc of the GM3 synthase expressed in the transfected cells of pM3T-7 were 270 μM and 313 μM, respectively (data not shown). These values were of similar magnitude to the results of Freuss et al. (28), i.e. 80 μM ($K_m$ for lactosylceramide) and 210 μM ($K_m$ for CMP-NeuAc), respectively. High specificity for lactosyl-ceramide was also similar, although a few acceptors besides lactosylceramide also showed low levels of activity in their results. On the other hand, the predicted molecular mass of the enzyme was 41,244 Da, whereas in their paper was 76 kDa in SDS-polyacrylamide gel electrophoresis. This discrepancy might be partially due to the glycosylation of the enzyme protein. Actually, the predicted amino acid sequence contained three possible sites of glycosylation at amino acids 180–182, 224–226, and 334–336 (Fig. 3). A similar discrepancy was also observed in the molecular mass of α2,3S-T activity in transfected cells. Furthermore, the deduced amino acid sequence indicated the presence of the sialyl motifs, which has been recognized among all mammalian and chicken sialyltransferases cloned so far (26), suggesting that this cDNA represents the GM3 synthase gene.

Northern Blot Analysis—Expression levels of the α2,3S-T gene in various tissues of mice were examined by Northern blots using total RNA. Among various tissues examined, brain showed the highest level, and then testis, heart and liver also showed relatively high levels, whereas almost all tissues showed some levels of the gene expression (Fig. 5). Mouse melanoma B78 also expressed a high level of transcript, consistent with the serological data indicating high levels of GM3 expression.

DISCUSSION

We have identified a cDNA that determines the expression of GM3 and induces α2,3S-T activity in transfected cells. Furthermore, the deduced amino acid sequence indicated the presence of the sialyl motifs, which has been recognized among all mammalian and chicken sialyltransferases cloned so far (26), suggesting that this cDNA represents the GM3 synthase gene. Examination of the sequence showed features in common with other α2,3-sialyltransferases. As shown in Fig. 6, the derived GM3 synthase sequence contained a number of characteristic regions commonly detected only in the sequences of ST3Gal α2,3-sialyltransferases (see enclosed boxes in sialyl motif L and S). These regions may be involved in the recognition of acceptor structures. The conserved YPE sequence was also noted by Sasaki in a recent review (27). In addition, the ST8Sia group showed a common sequence (NPS) in the carboxyl terminus region of the sialyl motif L. This sequence contrasted with the sequence YPE, which was commonly detected among ST3Gal group, supporting the idea that this region might be involved in the acceptor recognition.

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Among gangliosides, GM3 has the simplest carbohydrate structure and is located at the starting point of the synthesis of all ganglioside series gangliosides. Almost all mammalian tis-

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2 We propose here to assign the name of ST3Gal V to GM3 synthase.
sues and cells contain GM3 (1), suggesting its important biological role. Moreover, a number of studies have shown that GM3 functions in the regulation of platelet-derived growth factor receptor (29) and epidermal growth factor receptor (30–32) in cell adhesion (33–35), in the regulation of keratinocyte proliferation (36), and in the promotion of the differentiation of

FIG. 3. Nucleotide sequences of cloned α2,3S-T pM3T-7. Top, deduced amino acid sequences for the single open reading frame. The putative transmembrane region with 17 amino acids was double underlined. Candidates of N-glycosylation site were marked by a single underline. Bottom, hydropathy analysis of the coding region based on the deduced amino acids according to Hopp and Woods (40).
HL60 leukemia cells (37). Recently, Yamamura et al. (38) reported that GM3 co-localized with various signaling molecules such as c-Src and a low molecular GTP-binding protein Rho on the cell surface of B16 forming a glycolipid-enriched microdomain. Therefore, GM3 itself might play important roles at diverse situations for the regulation of cell proliferation and differentiation. Following the isolation of the GM3 synthase gene, the effects of GM3 endogenously generated in the cells can be studied to confirm the possible functions described above. Moreover, human cDNA of GM3 synthase has recently been cloned by Ishii et al. (39), enabling us to analyze the regulatory mechanisms of leukocyte differentiation by gangliosides.

Besides the importance of GM3 itself, α2,3S-T is also important, because it can regulate the amount of all gangliosides in particular tissues and cells. For example, although a majority of human melanoma cells characteristically express ganglioside GD3, they also express very high level of GM3. The high GD2 expression in neuroblastoma cells is also partly based on the high expression of GM3. Because GM3 expression is ubiquitous, its importance is often not appreciated. However, the quantity of GM3 is quite different among different normal tissues and various tumor cells. GM3 seems to control the expression levels of more specific and complex gangliosides by adjusting the amounts of precursor structures. Therefore, the mechanisms for the regulation of the gene expression of α2,3S-T are of great importance and interest.

Mammalian brain tissues usually express low levels of GM3 compared with the level of complex gangliosides. However, the actual synthesis of GM3 should be much higher than expected from the amount of GM3, because all complex ganglioside series gangliosides are synthesized through GM3. Results of Northern blotting clearly indicated that GM3 synthase takes place most actively in the brain among the normal tissues tested (Fig. 5). During the study of substrate specificity of the
cloned α2,3S-T, we found that GD1b specifically inhibited
the synthesis of GM3 with endogenous acceptor lactosylceramide
in membrane preparations (data not shown). This fact indicates
that later members of the biosynthetic pathway (GD1b in this
case) may regulate α2,3S-T activity by a feedback mecha-
nism in the brain tissue. The regulatory mechanisms of GM3
expression at the levels of gene expression and post-translation
are presently being investigated.

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