Cloning of a mouse β1,3 N-acetylgalactosaminyltransferase Lc3 synthase gene encoding the key regulator of lacto-series glycolipid biosynthesis*

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Running title: Cloning of a β3GnT Lc3-synthase

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Summary

The distinction between the different classes of glycolipids is conditioned by the action of specific core transferases. The entry point for lacto-series glycolipids is catalyzed by the β1,3 N-acetylglucosaminyltransferase Lc3 synthase enzyme. The Lc3 synthase activity has been shown to be regulated during development, especially during brain morphogenesis. Here, we report the molecular cloning of a mouse gene encoding an Lc3 synthase enzyme. The mouse cDNA included an open reading frame of 1131 bp encoding a protein of 376 amino acids. The Lc3 synthase protein shared several structural motifs previously identified in the members of the β1,3 glycosyltransferase superfamily. The Lc3 synthase enzyme efficiently utilized the lactosyl-ceramide glycolipid acceptor. The identity of the reaction products of Lc3 synthase transfected CHOP2/1 cells was confirmed by thin-layer chromatography immunostaining using antibodies TE-8 and 1B2 that recognize Lc3 (GlcNAc[β1,3]Gal[β1,4]Glc-ceramide) and nLc4 (Gal[β1,4]GlcNAc[β1,3]Gal[β1,4]Glc-ceramide) structures, respectively. In addition to the initiating activity for lacto chain synthesis, the Lc3 synthase could extend the terminal N-acetyllactosamine unit of nLc4, and also had a broad specificity for gangliosides GA1, GM1, and GD1b to generate neolacto-ganglio hybrid structures. The mouse Lc3 synthase gene was mainly expressed during embryonic development. In situ hybridization analysis revealed that the Lc3 synthase was expressed in most tissues at embryonic day 11 with elevated expression in the developing CNS. Postnatally, the expression was restricted to splenic B cells, the placenta, and cerebellar Purkinje cells where it colocalized with HNK-1 reactivity. This data supports a key role for the Lc3 synthase in regulating neolacto series glycolipid synthesis during embryonic development.
Introduction

Glycolipids are ubiquitous constituents of cellular membranes. The pattern of glycolipid expression varies according to cell type and is developmentally regulated. Functionally, glycolipids have been implicated in various biological processes related to cell activation (1;2), differentiation (3;4) and adhesion (5;6). Glycolipids also play important roles in infectious diseases, since they function as receptors for some viruses (7;8) and bacterial toxins (9;10). Moreover, cellular transformation is often accompanied by changes in the patterns of glycolipid expression (11;12), which may be of prognostic and diagnostic values.

Glycolipids can be classified according to their respective core oligosaccharide structures. In mammals, four major classes can be distinguished (13). Isoglobo-, globo-, lacto- and ganglio-series glycolipids are differentiated by the third saccharide unit added after the common lactosyl core (Fig. 1). This selective step is achieved by specific glycosyltransferase enzymes, which by their presence or absence in cells control the nature of the glycolipid repertoire. The molecular cloning of key glycosyltransferase genes allows a precise analysis of the dynamics of glycolipid expression and the possible correlation of changes in glycolipids with specific biological processes.

The β1,3 N-acetylglucosaminyltransferase Lc3 synthase is the key enzyme controlling the expression of lacto-series glycolipids. This class comprises both neutral and charged compounds and are characteristic glycolipids of human colonic carcinomas and leukemia cells (12;14). A number of sialosyl-, disialosyl-, fucosyl- and sulfoglucuronosyl-lactoglycolipids have been characterized in the nervous system (15;16). The pattern of expression of the HNK-1-


reactive sulfoglucuronylglycolipids (SGGL)\(^1\) and other neolactoglycolipids has been shown\(^4\) to be conditioned by the key Lc3 synthase activity (17). The Lc3 synthase enzyme is highly regulated during development, being most active by embryonic day 15 (E15) in the rat cerebral cortex and then decreasing to undetectable levels by postnatal day 10. In the adult cerebellum, the Lc3 synthase activity is restricted to Purkinje cells (18). Restoration of SGGL synthesis in primary granule neuron culture promotes neurite outgrowth and cell aggregation (19) suggesting that the control of SGGL expression by Lc3 synthase may have significance during development of the nervous system. In the present report, we describe the cloning of a Lc3 synthase gene and the characterization of the corresponding recombinant Lc3 synthase enzyme. Also, using \textit{in situ} hybridization, we provide evidence that the Lc3 synthase gene is broadly expressed in the nervous system and other tissues during mouse embryonic development, but is restricted to select cell types in adult. These findings extend our understanding of this important gene in glycosphingolipid biosynthesis, for which the human and rat orthologs were recently reported (20).

\(^1\) Bn, benzyl; Cer, ceramide; DIG, digoxigenin; E, embryonic day; GA1, Gal(β1,3)GalNAc(β1,4)Gal (β1,4)Glc-Cer; GD1b, Gal(β1,3)GalNAc(β1,4)[Sia(α2,8)Sia(α2,3)]Gal(β1,4)Glc-Cer; GM1, Gal(β1,3)GalNAc(β1,4)[Sia(α2,3)]Gal(β1,4)Glc-Cer; Lc3, GlcNAc(β1,3)Gal(β1,4)Glc- ceramide; nLc4, Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc- ceramide; octyl, O(CH\(_2\))\(_8\)CO\(_2\)Me; PBS, phosphate buffered saline; pNP, p-nitrophenyl; SGGL, sulfoglucuronylglycolipid; TUP, theoretical upper phase.
Experimental procedures

Cloning and expression of the Lc3 synthase gene  The human expressed sequence tag fragment AI589087 was retrieved from the EST division of GenBank as it showed significant similarity to the β1,3 galactosyltransferase-I, -II, -III, -V (21;22) and β1,3 N-acetylgalactosaminyltransferase-I (23) proteins. The mouse and human cDNAs were isolated from λ-phage brain cDNA libraries (CLONTECH) using a probing fragment derived from AI589087 generated by PCR with 50 ng human genomic DNA as template with the primers 5GGTGATGTAGCTGCCAAAGTC3 and 5ATCTTCATTAATCTGCAGTATATTTGAC3. The mouse cDNA was subcloned as a SalI-XbaI 1.8 kbp fragment into the pFastbac1 vector (Life Technologies) opened with the same enzymes, whereas the human cDNA was subcloned as a 1.2 kbp Dral-Dral fragment into pFastbac1 vector linearized with StuI. The recombinant enzymes were produced with the baculovirus-Sf9 insect cell system as previously described (21).

Glycosyltransferase activity assays - Donor and acceptor substrates were from Sigma, except for the O(CH2)8CO2Me-derivatized (octyl) acceptors, which were kindly provided by Dr. Markus Streiff (Novartis, Basel). Sf9 cells (5 x 10⁶) infected with wild-type or with recombinant baculoviruses were lysed at 72 h post infection in 1 ml of 2% Triton X-100 in phosphate buffered saline (PBS) on ice for 15 min. After removal of the nuclei by centrifugation, glycosyltransferase activity was assayed using 10 μl of cell lysate in 50 μl of 50 mM cacodylate buffer, pH 7.0, 20 mM MnCl2, 5% Me2SO, 0.75 mM ATP, 0.5 mM UDP-sugar donor substrate including 5 x 10⁴ cpm of the corresponding UDP-[14C]sugar (Amersham) and various acceptors. Quantification of the reaction products was performed as described (21).
Thin-layer chromatography immunostaining of Lc3 Synthase transfected cells – The fullength coding region of the mouse and human Lc3 synthase gene were excised from pFastbac1 by BamHI and XbaI digestion, and subcloned into pcDNA3 for transient transfection of CHOP2/1 cells. This CHO glycosylation mutant is defective in Golgi sialic acid transport and in the Mgat1 gene required for initiation of N-linked glycan chain synthesis (24). As a result of these modifications these cells have greatly reduced glycoconjugate sialylation and N-linked glycosylation. After 70 h, cells were harvested by scraping, hand homogenized in isopropanol: hexane: H₂O (55:25:20), and dried under nitrogen. The residue was partitioned in chloroform: methanol: 0.9% saline (20:10:6) and the lower phase was reextracted with theoretical upper phase (TUP)-saline (methanol: 0.9% saline: chloroform - 48: 47: 3). The lower phase was extracted twice more with TUP-saline and dried under nitrogen. To reduce contaminating phospholipids, the residue was subjected to alkaline hydrolysis overnight with 0.2 M NaOH, neutralized with HCl, and purified by reverse phase C₁₈-column chromatography. Glycolipid products were separated on aluminum high-performance thin-layer chromatography plates (E. Merck) using a solvent system of chloroform: methanol: 0.25% CaCl₂ (50:40:10) and immunostained using monoclonal antibodies TE-8 or 1B2 (each at 1: 5 dilution), essentially as described (25).

Northern blotting - The Lc3 synthase mRNA levels were detected using multiple tissues membranes of mouse adult tissue and embryonic stage RNA from Sigma. A 1.8 kbp SalI-XbaI fragment from the mouse Lc3 synthase cDNA was labelled with [α-³²P]CTP (Hartmann Analytics, Braunschweig, Germany) by random priming and hybridized to the poly(A)+ RNA blots. Blots were washed in 0.1 x SSC, 0.1% SDS heated up to 55 °C and exposed for four days between intensifying screens at -70 °C.
**In situ hybridization** - Digoxigenin (DIG)-labelled sense and antisense riboprobes were synthesized using a DIG RNA synthesis kit (Roche Molecular Biochemicals) from a 0.7 kbp HindIII-BglII fragment of the mouse Lc3 synthase cDNA subcloned into the plasmid pGEM3Zf(+) (Promega) following the instructions of the manufacturer. For *in situ* analysis of spleen Lc3 synthase expression, spleens from C57BL/6 mice aged of 8-12 weeks were frozen in Tissue-Tek (Miles, Elkhart, IN). Cryostat sections of 10 µm were cut at 20 °C and thaw mounted on Superfrost plus microscope slides (Faust, Germany). Sections were dried and fixed for 1 h in freshly prepared 4% paraformaldehyde in 0.1 M PBS pH 7.4 and acetylated for 10 min with 0.25% acetic anhydride in 1% triethanolamine. For *in situ* analysis of brain tissues (E14 and older) , deeply anesthetized C57BL/6 mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and further fixed overnight in 4% paraformaldehyde at 4 °C followed by immersion in 30% sucrose. Dissected samples were frozen on dry ice and sectioned at 25-35 µm thickness with a sliding microtome, then mounted on Superfrost plus microscope slides. Whole E11 embryos were prepared by direct overnight fixation in 4% paraformaldehyde followed by immersion in 30% sucrose. Cryostat cut sections of 16-20 µm thickness were prepared at -20° C and thaw mounted on Superfrost plus slides. After prehybridization and hybridization, the DIG-labelled RNA hybrids were detected with an anti-DIG Fab fragment conjugated to alkaline phosphatase (Roche Molecular Biochemicals) in a dilution of 1:2000. The color reaction was performed with nitrobluetetrazolim chloride and 5-bromo-4-chloro-3-indolylphosphate with levamisole added to block endogenous phosphatase activity.

**Immunohistochemistry** - Cryostat sections adjacent to those used for *in situ* hybridization were air-dried, fixed in acetone for 10 min and incubated for 5 min in 0.5% H$_2$O$_2$ in
methanol to quench endogenous peroxidase activity. Slides of spleen sections were first
incubated for 30 min in blocking solution (3% goat or rabbit serum in PBS), then incubated
for 30 min with primary antibodies diluted in blocking solution, followed by 30 min with
biotin-labelled secondary antibodies diluted in PBS. The slides were washed in PBS and
stained using the Vectastain ABC kit (Vector labs) with diaminobenzidine substrate
(Sigma). Rat anti-mouse CD19 monoclonal antibody MB19-1 (Pharmingen) was used as
primary antibody. The biotinylated rabbit-anti-rat secondary antibody was from Vector
labs. Comparative analysis of HNK-1 and calbindin localization in cerebellum sections was
performed by immunofluorescence analysis. Samples were blocked with 4% normal goat
serum for 2 hours, then incubated with HNK-1 hybridoma supernatant (1:5) and anti-
calbindin antibody (Sigma) diluted 1:50 in 1% NGS/0.2% Triton X-100 overnight at 4 °C.
HNK-1 and calbindin reactivity was localized with Goat anti-mouse IgM-Cy3 and Goat
anti-mouse IgG-Cy2 respectively (Jackson Immunoresearch).
Results

Several structural motifs are shared by the superfamily of β1,3 glycosyltransferases including β1,3 galactosyl- (21;26), β1,3 N-acetylglucosaminyl- (23;27) and β1,3 N-acetylgalactosaminyltransferases (28). This structural conservation allows the identification of possible additional β1,3 glycosyltransferase genes from genome databases. Using this approach, we have isolated a candidate gene encoding a protein showing 40 to 50% similarity with the query β1,3 glycosyltransferase proteins. The candidate mouse protein was 376 amino acid long, respectively, with a predicted transmembrane domain of 20 amino acids at the N-terminal region (Fig. 2). The human orthologous gene (GenBank accession AF368169), which we isolated from a brain cDNA library, coded for a protein of 378 amino acids displaying 77% identity with its mouse counterpart (data not shown). This cDNA was identical to the human β3Gn-T5 cDNA recently published by Togayachi et al. (20). The mouse and human cDNA were expressed as recombinant baculoviruses in insect Sf9 cells and assayed for glycosyltransferase activity. An N-acetylglucosaminyltransferase activity was detected toward several Gal-based acceptors, including significant utilization of lactosyl-ceramide Gal(β1,4)Glc-Cer (Table 1). This strong activity towards lactosyl-ceramide (1.23 nmoles/min/mg prot) suggested that this protein may represent an Lc3 synthase, the key enzyme in lacto-series glycolipid biosynthesis. By contrast, two previously cloned β1,3 N-acetylgalactosaminyltransferases had no significant activity towards lactosyl-ceramide. To confirm that this enzyme could function as an Lc3 synthase in vivo, the novel mouse cDNA as well as its human ortholog were overexpressed in CHOP2/1 cells, which are devoid of the Lc3 synthase activity and sialylation (19). Extracted glycolipids from cells transfected with a mock vector or vectors expressing the mouse and human cDNAs of interest were separated by high performance thin-layer chromatography. The Lc3 product was readily detected in
cells transfected with mouse and human cDNAs by immunostaining using the specific antibody TE-8 (Fig. 3). As expected, a significant portion of the Lc3 product was further modified by endogenous β1,4 galactosyltransferase activity to form paragloboside (Gal[β1,4]GlcNAc[β1,3]Gal[β1,4]Glc-Cer), which was localized by the 1B2 monoclonal antibody (Fig. 3). In contrast, significant levels of Lc3 and nLc4 were not detected in mock transfected cells. Thus, this experiment clearly shows that the cDNA clones encode Lc3 synthase enzymes.

The Lc3 synthase displayed a uniquely broad level of activity with a variety of galactose-terminated glycolipid acceptors. The Lc3 synthase utilized paragloboside with similar efficiency to lactosylceramide, demonstrating the ability to both initiate and extend lacto and neolacto chains. Unexpectedly, the enzyme also strongly utilized several gangliosides with exposed Gal(β1,3)GalNAc-R structures, including GA1, GM1, and GD1b (Table 2). Thin-layer chromatography analysis confirmed the addition of GlcNAc to each acceptor to form a single species (Fig. 4), which were strongly immunoreactive with mAb TE-8, specific for terminal GlcNAc(β1,3)Gal (data not shown).

To delineate the pattern of Lc3 synthase gene expression in vivo we have analyzed mRNA levels in mouse tissues by Northern blotting. Strong hybridization signals were detected in adult spleen and placenta, while faint signals were found in adult brain, lung, thymus and muscle tissues (Fig. 5). During development, the Lc3 synthase gene was maximally expressed by E11. Then, the mRNA levels progressively decreased but remained elevated up to the day of birth (Fig. 5).

The expression pattern of the Lc3 synthase was also localized by in situ hybridization with whole E11 embryonic mice (Fig 6A, B). Most tissues demonstrated significant expression of
Lc3 synthase mRNA, consistent with the intense signal detected at E11 by Northern analysis. Expression was relatively high in several regions of proliferative neuroepithelium in the developing CNS, including cerebral ventricular zones, which give rise to neurons of the adult cortex, and the neuroepithelium of the neural tube. Additionally, elevated expression was found in derivatives of the somitic mesoderm, including dorsal root ganglia and the sclerotome compartment which gives rise to the axial skeleton. The mandibular component of the first brachial arch, and the lens of the primordial eye (Fig. 6C, D) were also well stained at E11.

To investigate Lc3 synthase gene expression in the developing CNS in more detail, brain sections from later embryonic timepoints were examined. By E14 widespread expression was detected throughout the rostral caudal extent of the telencephalon (Fig 7A, B). Intense expression was maintained in the proliferative ventricular and subventricular zone, with the exception of the ventromedial wall of the caudal telencephalon (Arrows, Fig. 7A). This discontinuous ventricular expression pattern is very similar to that reported previously with monoclonal antibodies 7A in the mouse (29) and FORSE-1 in the rat (30). Both antibodies recognize fucosylated neolacto glycolipids bearing Lewis-X structures (3’-fucosyllactosamine), which are the predominant carriers of the Lewis-X determinant in the embryonic rodent cortex. At E17 additional staining in the expanding cortical plate became evident (Fig. 7C, D). At this timepoint, neurons migrate from the ventricular zone through the intermediate zone to form the characteristic laminar organization of the cortical plate in the adult forebrain. The accumulation of migrated cells from the strongly labeled ventricular zone may account for this signal. Substantial expression was also found in thalamic nuclei, the precerebellar neuroepithelium, and postmitotic neurons of the deep cerebellar nuclei (Fig. 7E, F). Several nuclei of the brainstem, including the inferior olive and pontine nuclei
also labelled strongly (not shown).

In the adult cerebellum, strong Lc3 synthase expression was found exclusively in Purkinje cell bodies in the Purkinje cell layer. The cell-specific expression pattern was confirmed by co-localization with the Purkinje cell-specific marker calbindin (31) (Fig. 8A). HNK-1 reactivity was confined to Purkinje cell bodies and their dendrites in the molecular layer, which precisely correlated with the Purkinje cell specific expression of the Lc3 synthase gene (Fig. 8B).

The Northern analysis of adult mouse tissues also suggested the spleen to be a potential site of lacto series glycolipid synthesis. In situ hybridization analysis of adult spleen revealed that Lc3 synthase positive cells were localized in the follicle region. The hybridization signal matched with B-lymphocyte populations as shown by CD19 immunostaining (Fig. 9). By contrast, splenic regions enriched in T-lymphocytes did not yield a hybridization signal with the Lc3 synthase antisense probe.
Discussion

We have reported here the molecular cloning of a β1,3 N-acetylglucosaminyltransferase gene coding for an Lc3 synthase enzyme involved in lacto-series glycolipid biosynthesis. The Lc3 synthase protein included several of the structural motifs identified previously in members of the β1,3 glycosyltransferase superfamily. This conservation suggests a common origin, indicating that a basic glycosyltransferase has likely evolved by gene duplication events, thereby allowing a qualitative expansion of oligosaccharide structures in higher organisms. In fact, structurally-related β1,3 glycosyltransferases are involved in nearly all types of glycoconjugate synthesis, including proteoglycan glycosaminoglycans, N-linked and O-linked glycans, and glycolipids.

Understanding the roles of specific classes of glycolipids in biological processes requires a clear view of the localization of these molecules in animal tissues. However, the in vivo detection of glycolipids themselves is hampered by the scarcity of reagents available that distinctly recognize specific structures. Furthermore, a selective detection of glycolipids can be difficult when the oligosaccharide moiety resembles those found on other classes of glycoconjugates. This last point is especially true for lacto-series glycolipids, which share several oligosaccharide structures with glycoproteins. Therefore, the availability of specific genetic probes, as represented by core-specific glycosyltransferase genes, constitutes an essential tools in the study of glycolipids in vivo. Along this line, the molecular cloning of the Lc3 synthase gene now enables a detailed investigation of the processes in which lacto-series glycolipids have been implicated. Noteworthy, the monoclonal antibodies recognizing lacto-series glycolipids such as TE-8 and 1B2 cannot be applied to detect the presence of the corresponding glycolipids in situ as they also recognize carbohydrate epitopes on glycoproteins.
Previous studies have documented the expression of Lc3 synthase enzyme activity as a key regulatory step in the synthesis of lacto series glycolipids in different tissues (12;32) and cell lines (12;33;3). This is also true for the developing brain where the expression of Lc3 synthase enzyme activity specifically regulates the expression of SGGLs (18). In situ colocalization of this new gene with HNK-1 reactive Purkinje cells supported its identity as the Lc3 synthase involved in SGGL synthesis. Additionally, the overlapping expression pattern of the Lc3 synthase with neolacto glycolipids in the embryonic telencephalon (29), and the lens of the developing rodent eye (35) indicates that this Lc3 synthase may be regulatory for expression of other brain neolactoseries glycolipids, as previously suggested from enzyme data (16).

The regulation of SGGL synthesis in neural tissue has been related to changes in morphological and adhesion properties of neurons (19). Experiments where Lc3 synthase gene expression can be controlled in cell culture models will enable the role of SGGL in these biological processes to be assessed. Likewise, the high level expression of Lc3 synthase in zones of the proliferating neuroepithelium in the developing neural tube, cerebral cortex, and cerebellar anlage suggests potential roles for this class of glycolipids in early neuronal development that require further investigation.

Earlier reports based on the study of cell lines showed that the Lc3 synthase activity was not expressed in lymphoid cells (34;36). However, our data clearly indicate that splenic B-lymphocyte express this gene. The discrepancy likely reflects differences in the glycolipid patterns between cells in vivo and transformed cell lines. Similar differences in glycolipid profiles between normal tissues and transformed cell lines have been noted in several other instances (11) and support the notion that in situ studies are better suited to investigate the physiological status of the glycolipid repertoire.
The mouse Lc3 synthase gene described here is the ortholog to the human cDNA published recently by Togayashi et al. (20). In the latter paper, the authors reported that the human gene is widely expressed based on RT-PCR data performed on adult tissue RNA and transformed cell lines. By contrast, our results in adult mouse tissues indicate that the Lc3 synthase gene expression is more restricted. This discrepancy likely reflects species differences. In fact, a Northern blot analysis of human adult tissue mRNAs revealed a broader pattern of Lc3 synthase expression (data not shown) than that detected in the mouse survey. However, we also cannot discount disparities accounted for by the different sensitivities of the respective detection systems or the difficult problem of genomic DNA amplification encountered with PCR quantitation of single exon genes from total RNA (20). The expression of Lc3 synthase in splenic B-cells is interesting in light of the high level of activity with gangliosides GA1, GM1 and GD1b. A rare species of hybrid ganglioside structures containing extended lactosamine structures on a GM1 core was previously identified in glycolipid extracts of splenic B-cells, but not T-cells (37). These were structurally identified as having the core structure GlcNAc(β1-3)Gal(β1-3)GalNAc-R. It seems likely that the Lc3 synthase is involved in the synthesis of this GlcNAcβ1,3-linkage. While the structural confirmation and significance of these await further analysis, these results may reveal a previously undescribed role for the Lc3 synthase in regulating synthesis of these unique glycolipids. The exact functions of lacto-series glycolipids have remained elusive with the exception of few specific processes. The disruption of the Lc3 synthase gene in mice should provide valuable insight into the functional involvement of this glycolipid class in developmental and physiological pathways.
Acknowledgments

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Figure legends

Figure 1. Biosynthesis of the major classes of glycolipids. The common lactosyl-ceramide (Gal[β1,4]Glc-Cer) structure is elongated by different glycosyltransferases, thereby defining the classes of glycolipids with their respective tri- or tetrasaccharide cores.

Figure 2. cDNA sequence and amino acid sequence of the mouse Lc3 synthase gene. The deduced amino acid sequence is shown in the single-letter code. The predicted transmembrane region is underlined and the motifs conserved in β1,3 glycosyltransferase proteins are shaded. The ATG translation initiation codon is marked in boldface.

Figure 3. Identification of the Lc3 synthase glycolipid products. Extracts of CHOP2/1 cells were separated by thin-layer chromatography and immunostained with the TE-8 and 1B2 antibodies. The left panel shows the migration or authentic glycolipid standards revealed by orcinol staining. The double band aspects of glycolipid species are caused by variation in the ceramide composition.

Figure 4. Extension of gangio-series acceptors by the recombinant Lc3 synthase enzyme. Thin-layer chromatography and orcinol staining of products obtained from reactions of the recombinant Lc3 synthase (+) with the lacto-series acceptors Lc2, nLc4 and the ganglio-series acceptors GA1, GM1, GD1b. Similar reactions performed with the lysate of mock-infected Sf9 cells are shown in comparison (-).

Figure 5. Expression of the mouse Lc3 synthase in adult tissues and embryos as determined by Northern blot analysis. Each lane represents about 2 μg of poly(A)+ RNA. At the left, the size of the RNA markers is indicated in kb.
**Figure 6. Expression of the Lc3 synthase gene during mouse development.** Sagittal sections of a mouse embryo by E11 and of the developing eye at the same stage hybridized with a Lc3 synthase antisense probe (A, C) and a sense probe (B, D), respectively. The numbers on A mark the following organs: 1, telencephalic vesicle; 2, midbrain tectum; 3, fourth ventricle; 4, olfactory pit; 5, medial nasal process; 6, mandibular component of first brachial arch; 7, superior ganglion of vagus nerve; 8, pharynx; 9, heart; 10, hepatic primordium; 11, intestine; 12, sclerotome compartment; 13, floor plate; 14, spinal cord; 15, dorsal (posterior) root ganglion. Abbreviations for (C, D): L, lens; NR, neural retina; RPE, retinal pigment epithelium. Note the staining in the sense control for the latter is caused by pigment granules and does not represent localized transcript.

**Figure 7. In situ localization of Lc3 synthase during embryonic brain development.** Sections were hybridized with antisense (A, C, E) and sense (B, D, F) Lc3 synthase probes. A, B; coronal sections through E14 telencephalon. C, D; Sagittal sections through E17 cerebellar primordium. E, F; Sagittal sections through caudal E17 telencephalon. CP, cortical plate; CPx, choroid plexus; DCN, deep cerebellar nuclei; IZ, intermediate zone; LV, lateral ventricle; M, medulla; PCN, precerebellar nuclei; SP, subplate; T, thalamus; VZ, ventricular zone.

**Figure 8. Restricted expression of the Lc3 synthase gene in adult mouse cerebellum.** Panels A and B show regions of mouse cerebellum at different magnifications. Sagittal sections through the medial aspects of mouse cerebellum probed with antisense (upper left) and sense RNA (upper right) to mouse Lc3 synthase. Lower panels show double label immunofluorescence of sections adjacent to the *in situ* sections stained with antibody to HNK-1 (lower left) and the Purkinje cell marker calbindin, localized with Cy3 and Cy2-
conjugated secondary antibodies, respectively. ML, molecular layer; PCL, Purkinje cell layer; IGCL, intermediate granule cell layer.

**Figure 9. Expression of the Lc3 synthase gene in splenic B-lymphocytes.** 10 µm sections of adult mouse spleens hybridized with Lc3 gene antisense RNA probe (top), sense RNA probe (bottom), and stained with the anti-CD19 B-lymphocyte marker antibody MB19-1 (middle).
Table 1. Acceptor substrate specificity of the Lc3 synthase enzyme and other β1,3 N-acetylglucosaminyltransferases. All acceptors were assayed at 5 mM except the (octyl)-derived acceptors, which were assayed at 2 mM and Gal(β1,4)Glc-Cer assayed at 0.25 mM.

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<td>Sf9 mock*</td>
</tr>
<tr>
<td>Gal(α1-OpNP)</td>
<td>6</td>
</tr>
<tr>
<td>Gal(β1-OpNP)</td>
<td>21</td>
</tr>
<tr>
<td>GalNAc(α1-OpNP)</td>
<td>8</td>
</tr>
<tr>
<td>GalNAc(β1-OpNP)</td>
<td>1</td>
</tr>
<tr>
<td>GlcNAc(α1-OpNP)</td>
<td>2</td>
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<tr>
<td>GlcNAc(β1-OpNP)</td>
<td>3</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>UDP-GalNAc</th>
<th>pmol/min/mg prot added</th>
</tr>
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<tbody>
<tr>
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<td>Sf9 mock*</td>
</tr>
<tr>
<td>Gal(α1-OpNP)</td>
<td>6</td>
</tr>
<tr>
<td>Gal(β1-OpNP)</td>
<td>4</td>
</tr>
</tbody>
</table>

* Lysate of Sf9 cells infected with a wild-type baculovirus.
lysate of Sf9 cells infected with recombinant baculovirus.

† Carbohydrate donor.
§ (octyl), O(CH2)8CO2Me.
§§ Lac-N-tetraose, Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc; Lac-N-neo-tetraose, Gal(β1-
4)GlcNAc(β1-3)Gal(β1-4)Glc.
Table 2. N-acetylglucosaminyltransferase activity of the Lc3 synthase enzyme toward complex glycolipid acceptor substrates.

<table>
<thead>
<tr>
<th>Acceptor substrate (0.25 mM)</th>
<th>Sf9 mock*</th>
<th>Lc3 synthase</th>
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</thead>
<tbody>
<tr>
<td>Gal(β1,4)Glc-Cer</td>
<td>nd§</td>
<td>1.23</td>
</tr>
<tr>
<td>Gal(β1,3)GalNAc(β1,4)Gal(β1,4)Glc-Cer</td>
<td>nd</td>
<td>2.54</td>
</tr>
<tr>
<td>Gal(β1,3)GalNAc(β1,4)[Sia(α2,3)]Gal(β1,4)Glc-Cer</td>
<td>nd</td>
<td>1.74</td>
</tr>
<tr>
<td>Gal(β1,3)GalNAc(β1,4)[Sia(α2,8)Sia(α2,3)]Gal(β1,4)Glc-Cer</td>
<td>nd</td>
<td>2.10</td>
</tr>
<tr>
<td>Gal(β1,4)GlcNAc(β1,3)Gal(β1,4)Glc-Cer</td>
<td>nd</td>
<td>1.21</td>
</tr>
</tbody>
</table>

* Lysate of Sf9 cells infected with a wild-type baculovirus.
lysate of Sf9 cells infected with recombinant baculovirus.
§ nd, not detected.
-60 TTCTACAGAGCTCTATAGCCCTGAAGAAGCAAGCAGCCATATACCCACAGACCAGAGAAC
1 ATGAGACTCTTGGTGTCAGCAAGAGTCAAAAAATGGAAGATTTTTTCTACTT
MRLFVSRVRKWKIFHEFVT
61 TGTTTTATATTAAGCTCTCATGTTTTTTTGGAGCCCAATCAGATATTACATCATGAGCCTAT
CFLSFMYVFSWSPINNYIMSH
121 ATGAGATCCTACTCTACAGATACTCCCTACATCAGGTAAAATAGCTATGGGCTTTGTAACAAATCTCCCTG
MKSYSYRLVNSYGFVNNSL
181 TTCCTCAAGCACAGCTGTGGCCCTCACTACCACCACATGTAACAGAGAAG
SLKHSVQPHYLPYLINHREK
241 TGTGAGGCTCAAGATGTCCTACTCTACACTTTATAAAGACTGCCCCTGAAGAAACTAGGGC
CQADVDVLLLFIKTAPENYG
301 CGACGTTCGTCAATCAGAAAAACTGCGGGGCAATGAGAATTGTTCAGTCTCAACTCAAT
RRSARIRKTWGNENYVQSLN
361 GCAACAACATCCAAAATCTGTGTTGCATTAGAACTCCTGCTGCTGGAAGGGAAAGAACTG
ANIKILFALGTGGGPKGLEL
421 CAAAAAGACTAATCGGGGAGATCAAGTCAAGAGATATAATACGCAAGATTTCATT
QKRLIGEDQVYKDLIQQDFI
481 GATCTGCTACAGAATCTTACTTCTAATAATTCTCTCTACAGTTCAGCTGGGCAATACCTTT
DSFHNLTSDKFQLFSWANTF
541 GTCACAGTGGCAGAAAATCTCTGATGACTGCTGATGATGATATATTATACAGCAAGATTTCCATT
VHMPPKFLMTADDDEFIHMPN
601 CTCAATGAAATACCTCAGGCTAGCAGAGTTGGAGCTGAGACCTGTCACCCCTCCCTTTTGATGATGCA
LIEMQLQEIGVYRDWIGH
661 GTCATCAGGCTGGCGGTCTCTCTGCTGAGAGAATAGCGCAATACATATAGTTCCCCTATGAAA
VHRGGPPVRRDKSSKYYVYPE
721 GTGTCAAGTGGCCAGCCTACCCCTGACTATACAGCTTGTTGCGCTATGTTGCTCAGAVA
VYKWPAVPDYTDAGAYVVS
781 GATGTAAGCTGCAAAATCTATGAGGCATCGCAGACAGCTAGAATCCAGATATGATAGATAG
DVAAKYEASQTNLSSMYID
841 GATGTAATCCAGGGCTCTCTGCAAAATAGGGGAATCTGGCAAGGACTGGTGATTGCTATT
DVFMGLCANKVGLPQDHVF
901 TTCCTGGGGGAGAAGGAAAATCTCTTATACCCCTGGCATCTATGAAAAGATGATGACATCT
FSEGKPYHPICYEKMMTS
961 CACGAGACTTACAAGATCTGCAGGACCTCCCTGAGATAAGGGCTCACATCCTAAGAAGATG
HGLQDLQDLWIEATHPKVK
1021 AACATTCAAAGGGTTTGGGGCTCAAATATACGCAAGTTAATAGATGTTCTC
NISKGFQGQIYCRCLIKIVLL
1081 TGCAAGCTAGCTTACAGGAAATCTCATACCCCTGGGCTGATTGCTTAGTAGTACATG
CRLTYRNSYPWCWAFA.
1141 ATGCCTGACTTGGTAAAGTGCATCGACCCAAAATTGGAATGGAAAATAAATACTGTAA
1201 ATGTTTTCTCTCCTACCTAAGTCAAATGAGGCAAAGAGATACCTTTCCAAACCCA
<table>
<thead>
<tr>
<th></th>
<th>Lc2</th>
<th>nLc4</th>
<th>GA1</th>
<th>GM1</th>
<th>GD1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Std</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

Lc3-

-GlcCer
-Lc2
-Gb3
-Gb4
Cloning of a mouse beta1,3 N-acetylglucosaminyltransferase Lc3 synthase gene encoding the key regulator of lacto-series glycolipid biosynthesis
Timothy R. Henion, Dapeng Zhou, David P. Wolfer, Firoze B. Jungalwala and Thierry Hennet

J. Biol. Chem. published online May 30, 2001

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