Glycobiology vol. 13 no. 6 pp. 445–455, 2003
DOI: 10.1093/glycob/cwg048

α1,3-Fucosyltransferase IX (Fut9) determines Lewis X expression in brain

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Received on September 11, 2002; revised on January 8, 2003; accepted on January 13, 2003

The expression of the Lewis X (Le^x) carbohydrate structure in brain is developmentally regulated and is thought to play a role in cell–cell interaction during neuronal development. Mice possess three functional *α1,3-fucosyltransferase* genes: *Fut4*, *Fut7*, and *Fut9*. Fut7 is known to have no activity to synthesize Le^x. In the present study, the relative activities of Fut4 and Fut9 for Le^x synthesis were determined using recombinant enzymes. Fut9 exhibited very strong activity for oligosaccharide acceptors and glycolipid acceptors, that is, more than 10- and 100-fold, respectively, than that of Fut4. Furthermore, both cerebrum and cerebellum at various stages of development (E17, P0, P7, P30, P100) expressed 15–100 times more *Fut9* transcript than *Fut4* transcript. Neurons and astrocytes in primary culture also expressed 10–15 times more *Fut9* than *Fut4* transcript. Moreover, α1,3-Fut activity toward a polylactosamine chain in homogenates of brain tissues and primary cultured cells showed a pattern typical of Fut9, not Fut4. The developmental profile of activity for the synthesis of Le^x was well correlated with that of *Fut9* transcript. Immunohistochemistry with anti-Fut9 monoclonal antibody revealed the distribution of the Le^x structure. These results showed that Fut9 is the most responsible enzyme for the synthesis of Le^x in brain.

Key words: α1,3-fucosyltransferase/Fut4/Fut9/Lewis X/SSEA-1

Introduction

Cell surface carbohydrates play important roles in cell–cell interaction and recognition. The Lewis X (Le^x) carbohydrate structure, α1,3-fucosyl-*N*-acetyllactosamine, is recognized as an antigenic epitope of stage-specific embryonic antigen-1 (SSEA-1) or CD15 antigen. The Le^x structure on the polylactosamine chain, which is detected as SSEA-1 at the morulae stage in mouse embryo, is considered to function as a cell–cell interaction ligand in the compaction process (Solter and Knowles, 1978; Gooi *et al*., 1981). Evidence has accumulated that the Le^x carbohydrate structure also functions as a cell–cell recognition molecule in the highly organized structures of the central nervous system (CNS) (Gotz *et al*., 1996; Sajdel-Sulkowska, 1998; Yoshida-Noro *et al*., 1999). The expression of Le^x in the brain of humans, monkeys, rodents, chickens, and *Xenopus* is developmentally regulated and region-specifically localized (Schonlau and Mai, 1995; Chou *et al*., 1996; Dasgupta *et al*., 1996; Gocht *et al*., 1996; Streit *et al*., 1996; Wiederschain *et al*., 1998; Allendoerfer *et al*., 1999; Mai *et al*., 1999; Shimoda *et al*., 2002). For example, in rodent CNS, Le^x is expressed in motor and auditory cortex, optic chiasm, hippocampus, cerebellum, brainstem, and spinal cord (Ashwell and Mai, 1997a,b; Marcus *et al*., 1999). This temporal and region-specific pattern of expression is consistent with the hypothesis that Le^x plays an important role in neuronal development. More recently, anti-Le^x monoclonal antibodies (mAbs) have been demonstrated to inhibit the adhesion of cerebellar granule cells to astrocytes in primary culture and block neurite outgrowth in explant culture (Sajdel-Sulkowska, 1998; Yoshida-Noro *et al*., 1999).

The Le^x structure in brain is carried on glycolipids, proteins, and proteoglycans. The carbohydrate chain carrying the structure is synthesized by a series of glycosyltransferases in the Golgi apparatus, the last step of Le^x synthesis being the transfer of a fucose to the *N*-acetylglucosamine (GlcNAc) of the type 2 chain (Galβ1-4GlcNAc) with an α1,3-linkage by α1,3-fucosyltransferases.

The α1,3-fucosyltransferases (α1,3FUTs or α1,3Futs) make up a family. We have cloned mouse *Fut9*, human *FUT9*, and rat *Fut9* (*Fuc-TIX*) (Kudo *et al*., 1998; Kaneko *et al*., 1999; Shimoda *et al*., 2002) in addition to the five known human *α1,3FUTs*, *FUT3* (*Fuc-TIII*) (Kukowska-Latallo *et al*., 1990), *FUT4* (*Fuc-TIV*) (Goelz *et al*., 1990; Kumar *et al*., 1991; Lowe *et al*., 1991), *FUT5* (*Fuc-TV*) (Weston *et al*., 1992a), *FUT6* (*Fuc-TVI*) (Koszdin and Bowen, 1992; Weston *et al*., 1992b), and *FUT7* (*Fuc-TVII*) (Natsuka *et al*., 1994; Sasaki *et al*., 1994). FUT3, FUT4, FUT5, FUT6, and FUT9 can synthesize the Le^x structure, but FUT7 cannot. The five human α1,3FUTs (FUT3, 4, 5, 6, 7) share a highly homologous sequence, whereas FUT9

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has a quite different sequence (Kaneko *et al.*, 1999). This indicated that the substrate specificity of FUT9 is unique among the α1,3FUTs. In fact, FUT9 preferentially transfers Fuc to the GlcNAc residue at the nonreducing terminal end of the polylactosamine chain, resulting in the terminal Le^x structure, whereas the other α1,3FUTs preferentially transfer a Fuc to the GlcNAc residue at the penultimate position, resulting in the internal Le^x structure (Nishihara *et al.*, 1999). Reflecting the substrate specificity, FUT9 exhibits 20-fold stronger activity for the synthesis of CD15, the terminal Le^x structure, than does FUT4, whereas FUT4 exhibits 4.5-fold stronger activity for the synthesis of VIM2, the internal Le^x structure, than does FUT9 (Nakayama *et al.*, 2001). FUT9 is mainly expressed in neuronal cells in the CNS, stomach epithelial cells, and peripheral blood leukocytes (Kaneko *et al.*, 1999; Nakayama *et al.*, 2001) and is considered the most likely candidate for the enzyme synthesizing Le^x in brain.

Mice have only three functional *α1,3Fut* genes, *Fut4*, *Fut7*, and *Fut9*, corresponding to the human *FUT4*, *FUT7*, and *FUT9* genes (Kaneko *et al.*, 1999). The other mouse *α1,3Fut (Fut3*, *5*, and *6*) genes were found to be pseudo-genes. Fut7 is limited to specific cells, such as leukocytes and endothelial cells of the venule, and cannot synthesize the Le^x structure (Maly *et al.*, 1996; Nakayama *et al.*, 2001). Fut4 is ubiquitously expressed in various tissues (Ozawa and Muramatsu, 1996; Kaneko *et al.*, 1999). Of the three functional mouse *α1,3Fut* genes, *Fut4* and *Fut9* are expressed in brain and both can synthesize the Le^x structure. Rat Fut4 and Fut9 are also expressed in brain and developmentally regulated (Baboval *et al.*, 2000). We recently demonstrated that *Pax6*, a transcription factor involved in brain patterning and neurogenesis, controls Le^x expression in the rat embryonic brain by regulating Fut9 (Shimoda *et al.*, 2002).

In the present study, we first performed a quantitative analysis of the relative activity to synthesize Le^x and the developmental expression of both mouse α1,3Futs based on transcriptional and enzymic activity levels. Then primary cultures of neurons or astrocytes and the brain tissues were also analyzed immunohistochemically. We concluded that Fut9 is most responsible for the expression of Le^x in brain.

Results

Relative α1,3-Fut activity of Fut9 and Fut4 for oligosaccharide substrates and glycolipid substrates

Both Fut4 and Fut9 are expressed in mouse brain. As reported previously, human FUT9 exhibits 20-fold stronger activity for the synthesis of CD15, the terminal Le^x structure, than does FUT4, whereas FUT4 exhibits 4.5-fold stronger activity for the synthesis of VIM2, the internal Le^x structure, than does FUT9 (Nakayama *et al.*, 2001).

We first determined the relative activity of mouse Fut4 and Fut9 to make sure they were the same as in humans. Neuro2A cells transfected stably with each of two *Fut* genes that were used as enzyme sources. To obtain the relative activity for Fut4 and Fut9, the amount of Fut4 and Fut9 expressed in each cell homogenate was adjusted judging from the transcript level, and the same amount of each was added to the enzyme reaction. Table I shows the relative α1,3-Fut activities of the two enzymes toward three 2-aminobenzamide (2AB)-labeled oligosaccharide substrates. A polylactosamine acceptor, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-2AB (3LN-2AB), has two GlcNAc residues to be fucosylated, the distal GlcNAc (V) and the internal GlcNAc (III). The activity of Fut9 toward the distal GlcNAc (V) was 90%, that is, 15.5 times stronger than the activity of Fut4 (5.8%), and was as high as the Fut9 activities toward Galβ1-4GlcNAcβ1-3Galβ1-4Glc-2AB, lacto-N-neotetraose-2AB (LNnT-2AB) (100%), and Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-2AB (2LN-2AB) (94%). Fut4 showed weak activity toward the distal GlcNAc (V) of 3LN-2AB (5.8%), which was as low as the Fut4 activities toward LNnT-2AB (6.6%) and 2LN-2AB (9.3%). In contrast, Fut4 showed stronger α1,3-Fut activity toward the internal GlcNAc (III) (19%) of 3LN-2AB than the distal GlcNAc (V). The relative activity for the internal GlcNAc (III) fucosylation of Fut9 to Fut4 was 0.51. These results demonstrated that Fut9 exerts 10–15 times stronger activity for the Le^x synthesis than Fut4 on the oligosaccharide substrates.

Table I. Relative α1,3-fucosyltransferase activities of Fut4 and Fut9 for 2AB-labeled oligosaccharides

Substrate		Relative activity (%) [a]		Ratio of activity
		Fut4	Fut9	Fut9/Fut4
LNnT-2AB				
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-2AB		6.6	100	15.2
2LN-2AB				
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-2AB		9.3	94	10.1
3LN-2AB				
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-2AB	V	5.8	90	15.5
V III	III	19	9.8	0.51

The GlcNac residues underscored were fucosylated by Fut4 and Fut9.
[a]The levels of each of the two Fut activities were normalized as to the amounts of transcripts determined by real-time RT-PCR. The relative activity (%) was shown when the Fut9 activity for LNnT-2AB was 100(%).

The same recombinant enzymes of Fut4 and Fut9 were also used to determine the relative α1,3-Fut activities for glycolipid acceptors. Five different glycolipid substrates were employed for the assays as shown in Table II. The same amounts of Fut9 and Fut4 and the same amount of each substrate were used for the enzyme reactions, so we could determine the relative activities of the two enzymes for each substrate. On thin-layer chromatography (TLC) analysis of reaction products that had incorporated [^{14}C]-Fuc into each glycolipid acceptor, Fut9 gave intense positive bands of fucosylated products except for the substrate NeuGcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-1′Cer (IV^3 NeuGcnLc$_4$) (Table II). In contrast, the Fut4 activity for these glycolipid acceptors was very weak. This is not due to the artificial inactivation of Fut4 because the same Fut4 lysate used in this experiment showed high activity against the oligosaccharide acceptor, 3LN-2AB, as already mentioned (Table I). Relative α1,3-Fut activities between Fut4 and Fut9 for various glycolipid acceptor substrates are summarized in Table II.

Table II. Relative α1,3-Fut activities of Fut4 and Fut9 for glycolipids

Substrate	Product	Relative activity (%)[a]		Ratio of activity
		Fut4	Fut9	Fut9/Fut4
nLc_4	$III^3FucnLc_4$	0.38	100	260
$IV^3GalnLc_4$	$IV^3GalIII^3FucnLc_4$	0.82	130	160
$IV^3NeuGcnLc_4$	$IV^3NeuGcIII^3FucnLc_4$	<0.20	<0.20	—
nLc_6	$V^3FucnLc_6$[b]	<0.20	25	>125
	$III^3FucnLc_6$[b]			
$VI^3NeuGcnLc_6$	$VI^3NeuGcIII^3FucnLc_6$	0.70	7.0	10

[a]The levels of each of the two Fut activities were normalized as to the amounts of transcripts determined by real-time RT-PCR. The relative activity (%) was shown when the Fut9 activity for nLc_4 was 100(%).
[b]The two fucosylated products with a single fucosylation from nLc_6, $V^3FucnLc_6$ and $III^3FucnLc_6$, could not be detected as separate bands.

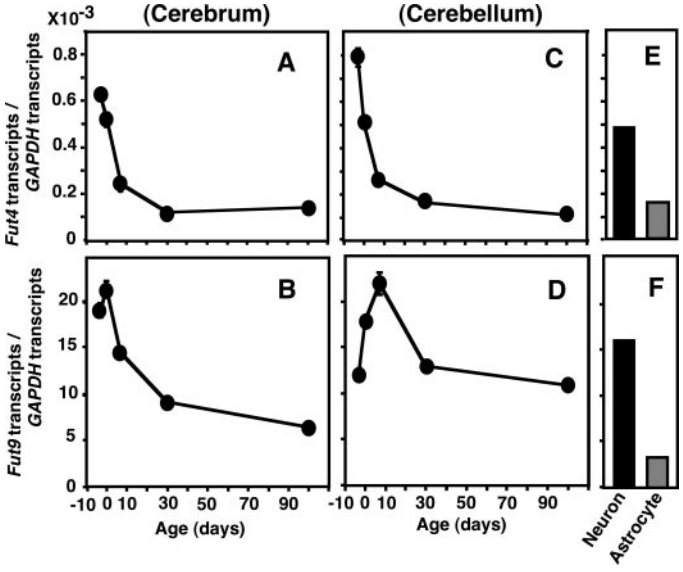

Fig. 1. Two α*1,3-fucosyltransferase* transcripts levels, *Fut4* (**A**, **C**, E) and *Fut9* (**B**, **D**, **F**), during mouse brain development and in primary culture. (**A** and **B**) Cerebrum at various stages of development (E17, P0, P7, P30, P100) expressed 30–80 times more *Fut9* than *Fut4* transcript. (**C** and **D**) Cerebellum including mesencephalon expressed 15–100 times more *Fut9* than *Fut4* transcript. (**E** and **F**) Both neurons and astrocytes in the cerebral primary culture expressed about 15 times more *Fut9* transcript than *Fut4* transcript. In neuron samples, more than 95% of the cultured cells were neuronal cells showing positive staining with anti-neurofilament mAb. In astrocyte samples, more than 95% of the cultured cells were astrocytes showing positive staining with anti-GFAP mAb.

The neutral fucosylated glycolipids carrying the Le^x structure, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc1-1′Cer ($III^3FucnLc_4$) and Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-1′Cer ($V^3FucnLc_6$), were apparently synthesized by Fut9. The two fucosylated products with a single fucosylation from the Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-1′Cer (nLc_6) substrate, which were $V^3FucnLc_6$ and Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc1-1′Cer ($III^3FucnLc_6$), could not be detected as separate bands. Galα1-3-Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer ($IV^3GalnLc_4$) was the best glycolipid acceptor for the transfer of Fuc by Fut9 (Table II). This was quite an interesting finding on Fut9 substrate specificity and contrasted with the negative activity of Fut9 toward $IV^3NeuGcnLc_4$. These results demonstrated that Fut9 cannot fucosylate the distal GlcNAc residue of the carbohydrate chain with the sialic acid at the nonreducing end but can efficiently fucosylate it when a galactose is localized in spite of the sialic acid. The short chain glycolipid, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer (nLc_4), was a better acceptor substrate than the longer chain glycolipid, nLc_6 (Table II). It was also interesting that Fut9 could synthesize the VIM-2 structure on the glycolipid, NeuGcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)-GlcNAcβ1-3Galβ1-4Glc1-1′Cer ($VI^3NeuGcIII^3FucnLc_6$), in which the internal GlcNAc residue was fucosylated, whereas Fut9 could not synthesize the sLe^x structure on the glycolipid, NeuGcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc1-1′Cer ($IV^3NeuGcIII^3FucnLc_4$).

Fut4 exhibited very weak activity toward various glycolipid acceptor substrates, as seen in Table II, although the same Fut4 lysates showed high levels of activity for the oligosaccharide acceptor, 3LN-2AB, as mentioned (Table I). The reaction products of Fut9 could be easily detected even when using the smaller amount of radioactive GDP-Fuc. We tried various reaction conditions to detect the α1,3-Fut activity of Fut4 for glycolipids but could detect only small amounts of fucosylated glycolipid products. For the distal GlcNAc on glycolipids, Fut4 could fucosylate only small amounts, less than 1/100 of those in the case of Fut9. Even for the internal GlcNAc of NeuGcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-1′Cer ($VI^3NeuGcnLc_6$), Fut4 was weaker than Fut9, with just 1/10 of the activity. We concluded that Fut4 is very weak at transferring Fuc to and synthesizing the Le^x structure on glycolipids, compared with Fut9. This strongly indicated that the Le^x structure on glycolipids in mouse brain is synthesized by Fut9.

Transcript levels of Fut9 *and* Fut4 *genes during mouse brain development*

Transcript levels of *Fut9* and *Fut4* during the development of mouse brain were determined quantitatively by the real-time reverse transcription polymerase chain reaction (RT-PCR) method. Developmental profiles of each transcript in cerebrum and in cerebellum containing mesencephalon are shown in Figure 1A–D. The vertical axes show the *Fut4* (Figure 1A, C) and *Fut9* (Figure 1B, D) transcript levels normalized with the transcript levels of glyceraldehyde phosphate dehydrogenase (GAPDH). The *Fut9* transcript was predominantly expressed at all stages both in cerebrum and in cerebellum containing mesencephalon. In cerebrum, *Fut9* was expressed in amounts 30–80 times that of *Fut4*. *Fut4* transcripts showed the highest value at stage E17 and decreased after birth (Figure 1A). *Fut9* transcripts increased

during the embryonic stage, showed the highest value at P0 and decreased (Figure 1B). The developmental profile of the *Fut9* transcripts well correlated with that of the activity to synthesize Le^x in cerebrum (Figure 2E).

In cerebellum and mesencephalon, *Fut9* was expressed at 15–40 times the amount of *Fut4* from the embryo to P0 stage and almost 100 times the level of *Fut4* after birth. *Fut4* transcripts showed the highest value at E17 and decreased after birth (Figure 1C). *Fut9* transcripts increased from the embryo to P7 stage, showed the highest value at P7, and decreased in adult brain (Figure 1D). The developmental change of *Fut9* transcripts in cerebellum and mesencephalon was also well correlated with that of activity for Le^x synthesis (Figure 2F).

From these four results—(1) that the α1,3-Fut activity of Fut9 for oligosaccharide substrates was more than 10 times that of Fut4, (2) that the α1,3-Fut activity of Fut9 for glycolipid substrates was more than 100 times that of Fut4, (3) that the expression level of *Fut9* was more than 15 times that of *Fut4* in brain throughout the course of development, and (4) that developmental profiles of *Fut9* transcripts in cerebrum and in cerebellum containing mesencephalon were well correlated with those for the synthesis of Le^x—we demonstrated that the Le^x structure in mouse brain is synthesized predominantly by Fut9, not by Fut4.

α1,3-Fut activity in mouse brain for polylactosamine acceptor, 3LN-2AB, shows a Fut9-specific profile

As already mentioned (Table I), we examined the specificity of mouse Fut4 and Fut9 for a polylactosamine chain, 3LN-2AB. The reaction products of the two enzymes had different high-pressure liquid chromatography (HPLC) profiles, as seen in Figure 2A and B. Fut4 gave a large P2 peak and small P1 and P3 peaks (Figure 2A), whereas Fut9 gave a large P1 peak and small P2 and P3 peaks (Figure 2B). The three products, P1, P2, and P3, were identified as

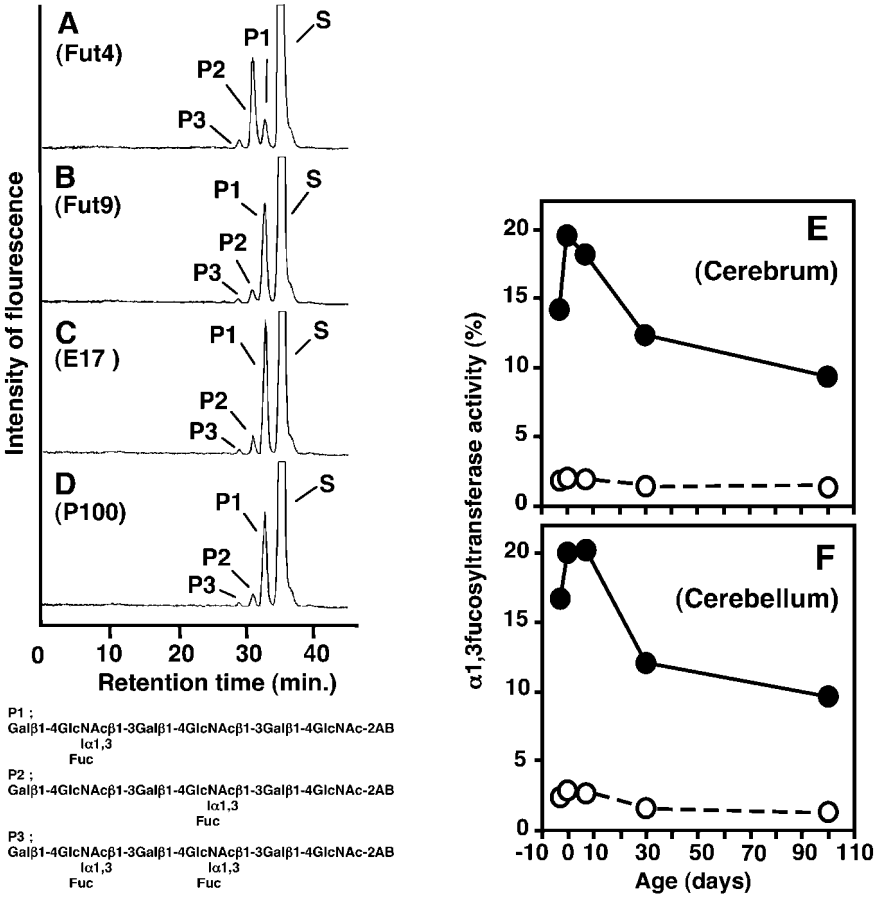

Fig. 2. Two α1,3-Fut activities, specifically Le^x (P1) and internal Le^x (P2) synthesizing activities, during mouse brain development. (**A**–**D**) Representative HPLC profile of α1,3-Fut products for 3LN-2AB. Enzyme sources were the homogenates of Neuro2A cells stably expressing Fut4 (**A**) or Fut9 (**B**) and the tissue extracts of E17 (**C**) or P100 (**D**) cerebrum. The three fucosylated products, P1, P2, and P3, were assigned in the previous study (Nishihara *et al.*, 1999) as follows: P1, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-2AB; P2, Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAc-2AB; P3, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAc-2AB; S, 3LN-2AB. (**E** and **F**) Developmental change of two α1,3-Fut activities, Le^x (P1) and internal Le^x (P2) synthesizing activities in cerebrum (**E**) and cerebellum including mesencephalon (**F**). The data are shown as the ratio of the fucosylated products (%) to the initial acceptor substrate. The percentage of P1 peak area (Le^x-synthesizing activity) and P2 peak area (internal Le^x-synthesizing activity) to the peak area of initial acceptor substrate is plotted with closed cycles and open circles, respectively.

Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc-2AB, Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAc-2AB, and Galβ1-4(Fucα1-3)-GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAc-2AB, respectively, in our previous study (Nishihara *et al*., 1999). Mouse Fut9 preferentially fucosylated the distal GlcNAc of 3LN-2AB, resulting in the product giving the large P1 peak that carries the Le^x structure, whereas mouse Fut4 preferentially fucosylated the internal GlcNAc to give a large P2 peak.

Representative HPLC profiles of the reaction products in tissue homogenates of mouse brain for 3LN-2AB are shown in Figure 2C and D. E17 (Figure 2C) and P100 (Figure 2D) cerebrum extracts showed the same product pattern as recombinant mouse Fut9 showed. The ratio of P1 to P2 of the α1,3-Fut activity in E17 and P100 cerebrum homogenates was the same as that of recombinant Fut9 products (Figure 2B–D). The other brain tissue samples, cerebrum of P0, P7, and P30 mice and cerebellum and mesencephalon of E17, P0, P7, P30, and P100 mice, also showed the same HPLC profile of fucosylated products as that of recombinant Fut9 (data not shown). These results confirmed the conclusion that the Le^x structure in mouse brain is synthesized predominantly by Fut9.

The α1,3-Fut activity to synthesize Le^x, for example, the activity for the P1 peak, in mouse brain was developmentally regulated. The activity of Le^x synthesis, corresponding to the P1 peak, was maximal at birth, at around P0 to P7, and decreased gradually in both cerebrum (Figure 2E) and cerebellum and mesencephalon (Figure 2F). Developmental profiles of the activity for Le^x synthesis were consistent with those of *Fut9* transcripts (Figure 1B, D). In contrast, the α1,3-Fut activity synthesizing the internal Le^x structure, that is, the activity for the P2 peak, was insignificant throughout the development of the brain.

These results demonstrated that the expression of Fut9 is developmentally regulated and directs the synthesis of Le^x in both cerebrum and cerebellum and mesencephalon, whereas Fut4 is only expressed at a very low level and is not responsible for the expression of Le^x in brain.

Specificity of an mAb against FUT9

The specificity of the mAb against human FUT9, KM2681, was determined by western blotting analysis (Figure 3). The cell lysates of Neuro2A cells transfected stably with each of two mouse *Fut* genes, *Fut4* and *Fut9*, which were used as recombinant enzyme sources. KM2681 showed specific reactivity against mouse Fut9 because of the highly conserved amino acid sequences between human FUT9 and mouse Fut9. KM2681 was used as anti-mouse Fut9 mAb in this article. KM2681 did not cross-react with mouse Fut4 and any other proteins included in Neuro2A cells.

Expression of Fut9 and SSEA-1 in primary-cultured neurons and astrocytes

Cerebral neuronal cells in primary culture were stained with anti-Fut9 mAb and anti-SSEA-1 mAb. The purity of the neurons in the culture was approximately 97%, as determined by immunostaining with anti-neurofilament antibody (Figure 4A). Fut9 was localized in the Golgi area of almost all neuronal cells (Figure 4B), but the Le^x structure recognized by anti-SSEA-1 mAb was not expressed in all neuronal cells (Figure 4C, D) because its expression is probably determined by some other glycosyltransferase(s) that synthesizes the root structure of the carbohydrate chain carrying the terminal Le^x structure. The surfaces of the body, processes, and growth cones of neuronal cells were strongly stained with anti-SSEA-1 mAb (Figure 4C).

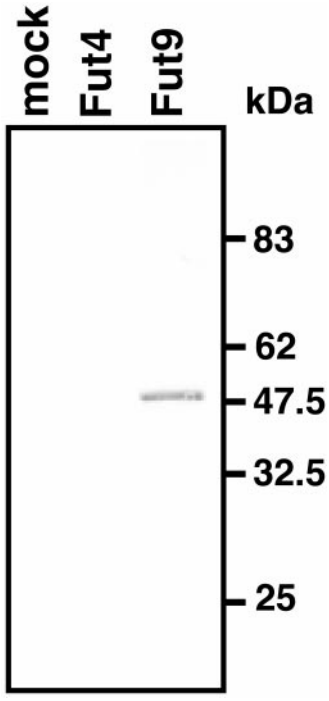

Fig. 3. Characterization of anti-FUT9 mAb by western blot analysis against the two kinds of the cells in which *Fut4* gene and *Fut9* gene were transfected. Mock, mock-transfected Neuro2A cells; Fut4, *Fut4* gene–transfected Neuro2A cells; Fut9, *Fut9* gene–transfected Neuro2A cells.

Cerebral astrocytes in primary culture were also stained with anti-Fut9 mAb and anti-SSEA-1 mAb. The purity of the astrocytes was over 98%, judging from the immunocytochemical-staining with anti-glial fibrillary acidic protein (GFAP) antibody. Fut9 was again localized in the Golgi area of almost all astrocytes (Figure 4F). But the Le^x structure recognized by anti-SSEA-1 mAb was not expressed in all astrocytes because its expression may also depend on precursor structures (Figure 4G, H). The surface of astrocytes was strongly stained by anti-SSEA-1 mAb (Figure 4G).

Quantitative analysis of Fut4 *and* Fut9 *transcripts in cerebral primary cultured cells*

Transcript levels of *Fut9* and *Fut4* genes in the neurons and astrocytes of the cerebral primary culture were determined quantitatively by real-time RT-PCR (Figure 1E, F). In both neurons and astrocytes, the *Fut9* transcript was predominant, at more than 15 times the level of *Fut4* transcript. This is consistent with the ratio of *Fut9* to *Fut4* transcript in mouse cerebrum tissue (Figure 1A, B). The neurons expressed five times more *Fut9* transcript than the astrocytes.

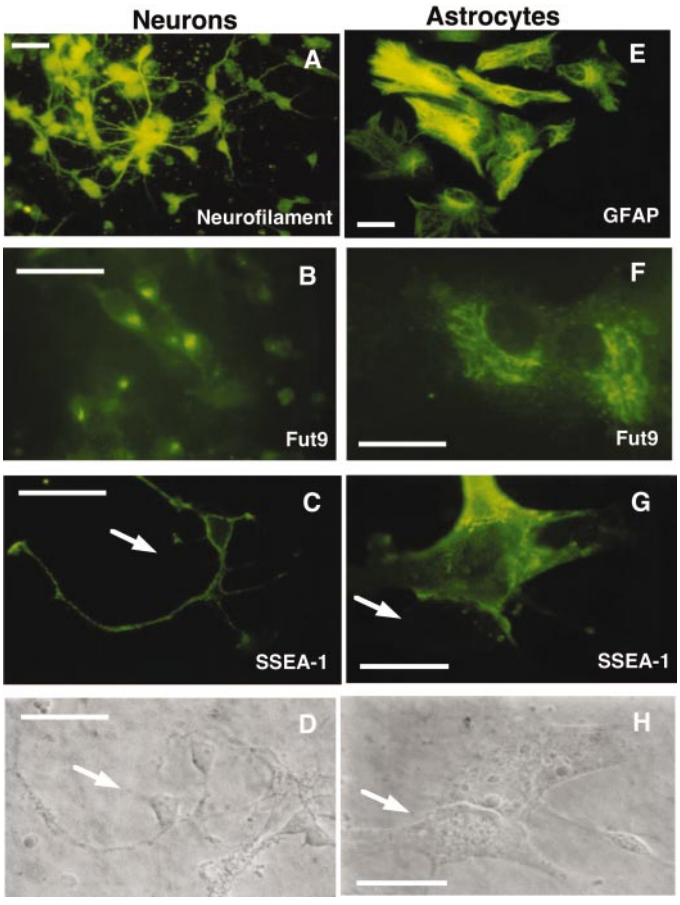

Fig. 4. Immunofluorescent staining of neurons and astrocytes in cerebral primary cultures with anti-Fut9 mAb and anti-SSEA-1 mAb. (**A**) Neurons with anti-neurofilament mAb. (**B**) Neurons with anti-Fut9 mAb. (**C**) (FITC) and (**D**) (phase contrast) neurons with anti-SSEA-1 mAb. (**A**–**D**) More than 97% of cultured cells were neurons that showed a positive staining with anti-neurofilament mAb. (**E**) Astrocytes with anti-GFAP mAb. (**F**) Astrocytes with anti-Fut9 mAb. (**G**) (FITC) and (**H**) (phase contrast) astrocytes with anti-SSEA-1 mAb. (**E**–**H**) More than 98% of cultured cells were astrocytes that showed positive staining with anti-GFAP mAb. Each scale bar indicates 20 μm.

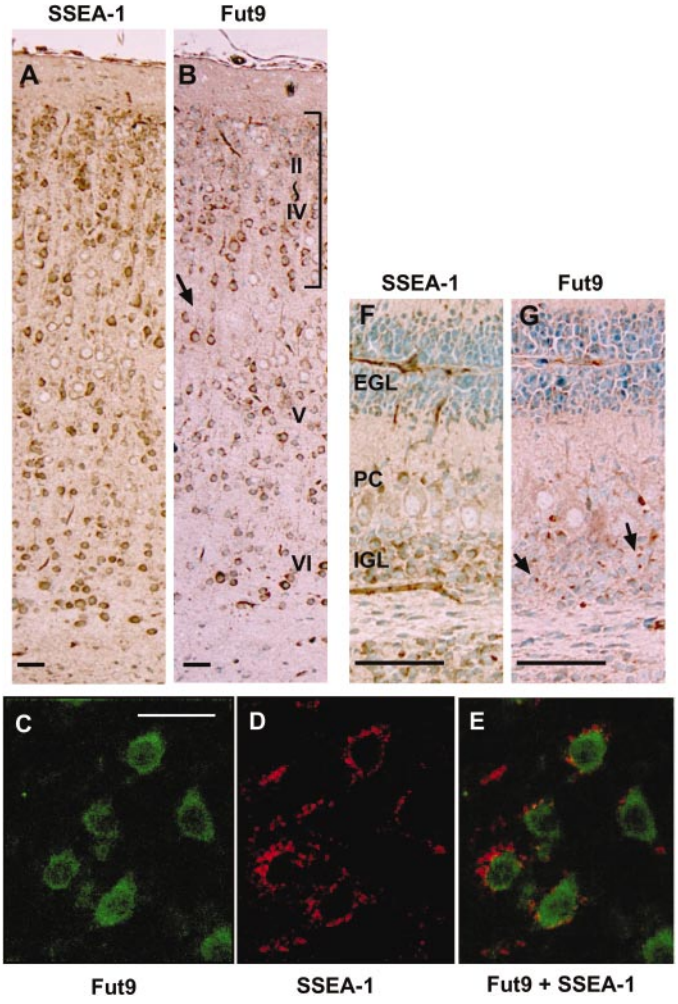

Fig. 5. Colocalization of Fut9 and Le^x in both cerebrum and cerebellum. (**A** and **B**) Immunohistochemical staining of P7 mouse cerebrum with anti-SSEA-1 mAb (**A**) and anti-Fut9 mAb (**B**). Frozen sagittal sections were used for immunohistochemical staining. Scale bar indicates 50 μm. (**C**–**E**) Confocal images of P7 mouse cerebrum double stained with anti- Fut9 mAb (**C**, FITC) and anti-SSEA-1 mAb (**D**, rhodamine). Fused image (**E**) showed that Fut9 and SSEA-1 localized in the same cells. Scale bar indicates 20 μm. (**F** and **G**) Immunohistochemical staining of P7 mouse cerebellum with anti-SSEA-1 mAb (**F**) and anti-Fut9 mAb (**G**). Frozen sagittal sections were used for immunohistochemical staining. Scale bar indicates 50 μm.

Immunohistochemical staining of P7 mouse brain with anti-Fut9 mAb and anti-SSEA-1 mAb

In the previous section (Figure 2), P7 mouse brain showed the highest α1,3-Fut activity for the synthesis of Le^x, that is, the highest P1 peak, during development. Sagittal sections of P7 mouse brain tissues were immunohistochemically stained with anti-Fut9 mAb and anti-SSEA-1 mAb. The immunohistochemical procedure was performed on frozen sections to detect both glycolipids and glycoproteins carrying the Le^x structure.

In cerebral cortex, pyramidal neurons in layers II–IV and those in layers V and VI were strongly stained by anti-SSEA-1 mAb (Figure 5A). Fut9 was detected also in the neurons in layers II–IV and those in layers V and VI (Figure 5B). Then we performed double staining with anti-SSEA-1 mAb and anti-Fut9 mAb for P7 mouse brain. Confocal images of pyramidal neurons in layers II–IV of the cerebral cortex double stained with anti-Fut9 mAb (fluorescein isothiocyanate; FITC) and anti-SSEA-1 mAb (rhodamine) are shown in Figure 5C and D, respectively. A fused image (Figure 5E) demonstrated that Fut9 and SSEA-1 localized in the same cells. Colocalization of Fut9 and Le^x was also observed in pyramidal neurons in layers V and VI of the cerebral cortex.

In cerebellum, granule neurons in the internal granule cell layer were stained strongly by both mAbs, whereas the granule neurons in the external granule cell layer were not stained. Notably, the granule neurons migrating from the external to the internal granule cell layer began to be stained by anti-SSEA-1 mAb (Figure 5F) and anti-Fut9 mAb (Figure 5G). Purkinje cells were faintly stained by both mAbs.

The colocalization of Fut9 and Le^x in both cerebrum and cerebellum again supported that the Le^x structure in mouse brain is synthesized mainly by Fut9.

Discussion

We conclude that the Le^x structure in mouse brain is synthesized mainly by Fut9 for the following reasons. (1) Fut9 exhibited more than 10 and 100 times the α1,3-Fut activity for oligosaccharide substrates and glycolipid substrates, respectively, that Fut4 exhibited. (2) *Fut9* was expressed in both cerebrum and cerebellum and mesencephalon at a level more than 15 times higher than that of *Fut4* throughout the development. (3) Extracts from cerebrum and from cerebellum and mesencephalon at each developmental stage showed the substrate specificity pattern that is unique to Fut9. (4) The developmental profile of Le^x synthesis is well correlated with that of *Fut9* transcript. Finally, (5) Fut9 colocalized with the Le^x structure in mouse brain. The high expression level of *Fut9* transcript in the brain and the strong relative activity of Fut9 for the synthesis of Le^x indicated that Fut9 contributes to the synthesis in brain with at least 15×10 times (150 times) more activity than Fut4. Fut9 must be the most responsible enzyme for the synthesis of Le^x in brain not only on glycolipids but also on glycoprotein and proteoglycan. The contribution of Fut4 to the synthesis of Le^x in brain would be small.

This developmental profile of α1,3-Fut activity and *Fut9* transcripts in mouse brain was consistent with a previous report on rat brain (Wiederschain *et al*., 1998), although in that study the site specificities of the activity for polylactosamine were not examined. The sudden disappearance of the Le^x-carrying glycolipids after birth (Jungalwala, 1994) did not correlate with the gradual decrease in the α1,3-Fut activity for Le^x synthesis (Figure 2E, F). As reported (Dasgupta *et al*., 1996), the Le^x expression on glycolipids in brain is determined by lactosylceramide *N*-acetylglucosaminyltransferase (Lc_3 synthase), which synthesizes the root structure of the glycolipids carrying Le^x. The marked decrease in the Lc_3 synthase activity after birth was reported to be the cause of the sudden disappearance of the Le^x-carrying glycolipids (Jungalwala, 1994). In fact, we recently cloned a gene encoding Lc_3 synthase and demonstrated that its expression level is well correlated with that of Le^x-carrying glycolipids during the development of the mouse brain (Togayachi *et al*., 2001).

In the present study, mouse Fut9 showed the same unique acceptor specificity for polylactosamine, that is, the preferential fucosylation of the distal GlcNAc residue, as human FUT9. Mouse Fut4 preferentially fucosylated the internal GlcNAc, like human FUT3, FUT4, FUT5, and FUT6. This acceptor substrate specificity corresponds to the position on the phylogenetic tree of α1,3-Fut members. According to this tree, the *Fut9* gene subfamily diverged from the ancestral gene before the other α1,3-Fut subfamilies. Mouse Fut9 exhibited much stronger activity for synthesis of Le^x on both oligosaccharides and glycolipids than mouse Fut4. Human FUT9 also exhibited much higher levels of activity for the synthesis on oligosaccharides (Nakayama *et al*., 2001) and glycolipids (data not shown) than human FUT4.

The mutant LEC11 cells, derived from Chinese hamster ovary cells, express an *α1,3-Fut* gene orthologous to the human *FUT6* gene but not the human *FUT9* gene (Zhang *et al*., 1999). LEC11 cells exhibited preferential transfer of Fuc to the internal GlcNAc of the nLc_6 neolacto-glycolipid acceptor (Zhang *et al*., 1999). In contrast, another mutant cell line, LEC12, expressed the hamster *Fut9* gene but not the hamster *Fut6* gene and exhibited preferential transfer of Fuc to the distal GlcNAc of nLc_6 (Zhang *et al*., 1999). In the present study, we could not separate the two fucosylated products $V^3FucnLc_6$ and $III^3FucnLc_6$. Considering the site specificity of the mouse Fut9 activity for the polylactosamine acceptor, 3LN-2AB, and the LEC12 activity reported previously (Patnaik *et al*., 2000), $V^3FucnLc_6$ must be a main product that is synthesized from nLc_6 by mouse Fut9.

Sherwood and Holmes (1999) reported that human recombinant FUT4 has low α1,3-Fut activity for neolacto-series glycolipid acceptor, nLc_4, $IV^3NeuGcnLc_4$, nLc_6, and $VI^3NeuGcnLc_6$, although they did not determine the amount of FUT4 and the activity of FUT4 relative to the other α1,3FUTs. Baboval *et al*. (2000) reported that rat recombinant Fut9 has much higher α1,3-Fut activity for glycolipid acceptor, nLc_4, than rat Fut4, although they did not determine the amount of rat Fut4 and rat Fut9 and the activity of rat Fut9 relative to the rat Fut4. In the present study, we demonstrated that the activity of mouse Fut4 toward neolacto-series glycolipid acceptors was very weak in comparison to the activity of mouse Fut9.

One interesting finding was that Fut9 can efficiently transfer a Fuc to the α1,3-galactosylated lactosamine chain of glycolipids, resulting in the synthesis of IV^3Gal $III^3FucnLc_4$. The Le^x structure, which is referred to as SSEA-1 in the embryo, is also expressed in undifferentiated mouse F9 teratocarcinoma cells, but the expression is lost in these cells when differentiation is induced with retinoic acid (Kudo and Narimatsu, 1995). The disappearance of Le^x in the differentiated F9 cells is caused by the up-regulation of α1,3-galactosyltransferase, which masks the Le^x structure by the addition of galactose with an α1,3-linkage (Cho *et al*., 1996). The highly effective production of IV^3Gal $III^3FucnLc_4$ by Fut9 (Table II) indicated that Fut9 does not compete with α3GalT for acceptor substrates and the Galα1-3Galβ1-4(Fucα1-3) GlcNAc-R structure can be synthesized in cells that express both Fut9 and α3GalT. This is in contrast to the absence of activity of Fut9 for α2,3-sialylated neolacto-glycolipid acceptor, $IV^3NeuGcnLc_4$ (Table II). Although Fut9 cannot transfer a Fuc to the distal GlcNAc residue of sialylated lactosamine—that is, it has no activity for sLe^x synthesis—it was found in the present study that Fut9 can synthesize the VIM-2 (CDw65) structure by α1,3-fucosylation of the internal GlcNAc of sialylated polylactosamine, as demonstrated by the activity for 3LN-2AB (Table I) and by the production of $VI^3NeuGcnLc_6$ (Table II).

Of the three functional mouse α1,3Futs, Fut4, -7, and -9, only Fut9 has an amino acid sequence that is highly conserved in the human counterpart FUT9, the level of conservation being equal to that for α-actin, suggesting a strong selective pressure for the preservation of the FUT9 (Fut9) sequence during evolution. In mouse, the Le^x structure appears on the surface of neural tubes at E9, and the

forebrain, the midbrain, and the spinal cord all begin to be stained by anti-Le^x mAb from E10 (Yamamoto *et al.*, 1985; Ashwell and Mai, 1997a). In chick embryo, the Le^x structure was observed in the elevating neural plate and neural fold at stage 7/8 and in the closing neural tube at stage 10/11 (Streit *et al.*, 1996). Moreover, there is evidence to suggest that the Le^x carbohydrate structure functions as a cell–cell recognition molecule in the highly organized structures of the CNS (Gotz *et al.*, 1996; Sajdel-Sulkowska, 1998; Yoshida-Noro *et al.*, 1999). The Le^x structure synthesized by Fut9 should play an important role in neurogenesis.

The synthesis of the HNK-1 structure, sulfoglucuronyl carbohydrate (SO_3^-–3GlcAβ1-3Galβ1-4GlcNAcβ1-R), may compete with the synthesis of the Le^x structure (Galβ1-4[Fucα1-3]GlcNAcβ-). A number of studies have suggested that the HNK-1 structure itself is involved in cell–cell recognition and signaling during the development of the CNS (for example, Nair *et al.*, 1993). Recently, the synthesis of HNK-1-carrying glycolipids in mature granule neurons was found to cause dedifferentiation, cell aggregation, and enhanced proliferation of neurites (Chou *et al.*, 1998). In rat cerebral cortex, both glycolipids and glycoproteins carrying HNK-1 are strongly expressed during the embryonic stage. In rat cerebellum, glycolipids carrying HNK-1 are expressed in a biphasic manner, with the initial peak at around P1-P3 and the second peak at P20 (Jungalwala, 1994). The stage of initial decline in the level of these glycolipids correlated with that of the migration of immature granule neurons from the external to the internal granule cell layer, guided by Bergman glial fibers.

We observed that the disappearance of the HNK-carrying glycolipids in those cells was correlated with the appearance of positive signals obtained with anti-SSEA-1 and anti-Fut9 antibodies. As seen from the immunohistochemical staining of P7 mouse cerebellum with anti-SSEA-1 mAb and anti-Fut9 mAb (Figure 5F, G), both the migrating granule neurons and the granule neurons in the internal granule cell layer expressed Fut9 and the Le^x structure. Baboval *et al.* (2000) reported that rat *Fut9* transcripts are expressed strongly in the cells of the internal granule cell layer in adult rat cerebellum. Mai *et al.* (1995) reported that the expressions of CD15 and HNK-1 are complementary in some regions of the brainstem and the cerebellum in the adult mouse. These results indicate that Fut9 regulates the expression of CD15 and HNK-1 positively and negatively, respectively, in mouse cerebellum. We will begin double staining with the anti-CD15 and anti-HNK-1 mAbs in brain tissue and primary-cultured cells. The biological functions of the Le^x and HNK-1 structures, whose expressions are probably under reciprocal regulation during neurogenesis, remain to be elucidated.

Materials and methods

Preparation of oligosaccharide and glycolipid acceptor substrates

LNnT was purchased from Oxford Glycosystems (Oxford, UK). 2LN and 3LN were prepared from keratan sulfate (Seikagaku-Kogya, Tokyo) as described in a previous paper (Nishihara *et al.*, 1999). These three oligosaccharide acceptor substrates were fluorescently labeled with 2AB according to the manual of the Signal 2AB glycan labelling kit (Oxford Glycosystems).

nLc_4 and $IV^3GalnLc_4$ were obtained from the neutral glycolipid fraction of bovine erythrocyte membranes. IV^3 $NeuGcnLc_4$ and $VI^3NeuGcnLc_6$ were purified from the acidic glycolipid fraction of the same erythrocyte membranes (Uemura *et al.*, 1978; Watanabe *et al.*, 1979). After the separation of neutral and acidic glycolipids using DEAE-Sephadex A-25 (Pharmacia Biotech, Tokyo), individual glycolipids were obtained by HPLC on a column of Iatrobeads (6RS-8060, Iatron, Tokyo) (Ledeen and Yu, 1982). nLc_6 was prepared from VI^3 $NeuGcnLc_6$ by neuraminidase treatment of *Arthrobactor ureafaciens* (Nacalai Tesque, Kyoto, Japan), followed by extraction with chloroform/methanol (2:1 and 1:1, v/v). All glycolipids used were checked by TLC, and their identities were confirmed.

Establishment of Neuro2A cells transfected stably with the Fut4 *or* Fut9 *gene*

The DNA fragments encoding the full-length open reading frames (ORFs) of the mouse *Fut4* and *Fut9* genes were isolated and subcloned into another expression vector, pCXN2, for expression in Neuro2A cells. Stable transformant cells were then selected using geneticin (1.2 mg/ml) (G418; Sigma, St. Louis, MO). The transcript levels of *Fut4* and *Fut9* genes were determined for each stable transformant by real-time RT-PCR, as described in a following section.

Determination of the relative α1,3-Fut activity of Fut9 and Fut4 for oligosaccharide and glycolipid acceptor substrates

The levels of *Fut4* and *Fut9* transcripts expressed in stable transformant cells were measured, and homogenates of the cells were used as an enzyme source for assay of α1,3-Fut activity using each acceptor substrate. To obtain the relative activity for Fut4 and Fut9, the amount expressed in each cell homogenate was adjusted judging from the transcript level, and the same amount of each was added to the enzyme reaction. The same amount of each substrate was also added. The enzyme reaction and the HPLC analysis of reaction products were performed as described in a previous paper (Nishihara *et al.*, 1999).

Using 10 μg of each glycolipid acceptor, the α1,3-Fut reaction was allowed to proceed at 37°C for 2 h in 50 mM 4-morpholine propane sulfonic acid (pH 7.5), 10 mM ATP, 100 mM NaCl, 5 mM $MnCl_2$, 10 mM GDP-[^{14}C]Fuc (4.4×10^5 cpm/nmol), and 0.2% Triton X-100 and terminated by boiling for 3 min followed by the adding of 0.1 M KCl. The enzyme sources were the same as for the oligosaccharide acceptor substrates, and the amounts of Fut4 and Fut9 in the reactions were adjusted according to the level of transcript. After centrifugation of the reaction mixtures, the supernatant was applied to a Sep-Pak C_{18} column (Waters, Milford, MA) equilibrated with 0.1 M KCl. The unreacted GDP-Fuc was washed out with H_2O, and the lipid products were eluted with methanol. The eluates were dried with an N_2 evaporator and dissolved in 30 μl

methanol. The glycolipid products (10 μl) were applied to a HPTLC plate (Merck, Darmstadt, Germany) and developed in chloroform-methanol-0.02%$CaCl_2$ (5:4:1). The bands of reaction products incorporating radioactivity were detected with a BAS2000 Imaging Analyzer system (Fujifilm, Tokyo). III^{3}FucnLc$_4$, neutral glycolipid mixture, and ganglioside mixture (DIA-IATRON, Tokyo) were used as TLC standards.

Quantitative analysis of Fut4 and Fut9 transcripts in brain tissues and primary cultured cells using the real-time RT-PCR method

Total cellular RNA was isolated from mouse brain tissues and primary cultured cells using the acid guanidium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). Complementary DNA was synthesized with an oligo dT primer from the total RNA using a SUPERSCRIPT Preamplification System for First Strand cDNA Synthesis (Invitrogen, NY). The cDNAs were used for the real-time RT-PCR.

The real-time RT-PCR was carried out using a TaqMan PCR kit (Roche, Piscataway, NJ) and the following gene-specific primer sets: *Fut4* forward primer, CAAAGCCCTGGAGACCGT AGGT; *Fut4* reverse primer, CGCTCCTGGAATAGAGGAAGCC; *Fut9* forward primer, CA AATCCCATGCGGTCCTGAT; *Fut9* reverse primer, TGCTCACCGTCAAGAAGC CATAA; GAPDH forward primer, TCCTGCACCACCAACTGCTTAGCCC; and GAPDH reverse primer, CTTGATGTCATCATAYTTGGCAGG. The gene-specific probes, CCTCCCAT ACTCCAGGGCTGCGGG, CCTCAGCAGGCCAGGCCACCCTTT, and TGACCACAGTCCATGCCATCACTGC for *Fut4*, *Fut9*, and GAPDH, respectively, were rabeled with 5′-FAM and 3′-TAMRA. After the mixing of each solution, 50 cycles of PCR were performed in ABI PRISM 7700 (Applied Biosystems Japan, Tokyo) under the following conditions: 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Standard DNA plasmids of the respective full-length ORF cDNA were used for the quantitation of each transcript.

Assay of α1,3-Fut activity for the polylactosamine chain in brain tissue extracts

The cerebrum and the cerebellum containing mesencephalon were each sampled at several developmental stages (E17, P0, P7, P30, and P100) and frozen at −70°C until use. The tissues were crushed and solubilized in HEPES buffer (pH 7.2) containing 2% Triton X-100 by brief sonication, and 50 μg of extracted protein was subjected to the assay of activity for polylactosamine acceptor, 3LN, as described previously (Nishihara *et al*., 1999).

Establishment of a mAb against FUT9

The ORF of the human *FUT9* gene was subcloned into a bacterial expression vector, pET-14b (Novagen, Madison, WI). Mice were immunized with the recombinant protein expressed in *Escherichia coli*. The screening of hybridomas reacting to human FUT9 was performed using microtiter plates coated with the recombinant human FUT9 to obtain anti-FUT9 mAb, KM2681 (mouse IgG1).

Western blotting analysis

Neuro2A cells transfected stably with each of two mouse *Fut* genes, *Fut4* and *Fut9*, were used as recombinant enzyme sources. Cell pellets were solubilized in 20 mM HEPES buffer (pH 7.2) containing 2% Triton X-100 by brief sonication. Proteins separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis were transferred to an Immobilon polyvinylidene difluoride membrane (Millipore) in a Transblot SD cell (Bio-Rad, Hercules, CA). The membrane was blocked with phosphate buffered saline (PBS) containing 5% skim milk at 4°C overnight and then incubated with 10 μg/ml of anti-FUT9 mAb, KM2681. The membrane was stained according to the manual with the ECL western blotting detection reagents (Pharmacia Biotech, Tokyo).

Primary culture of neurons and astrocytes from mouse brain

E17 and P0 mouse brain was used for the primary culture of neurons and astrocytes, respectively, according to a method described previously (Nagata *et al*., 1993). The brains were cleaned of meninges and blood vessels, minced, and trypsinized in PBS containing 0.25% trypsin and 0.05% DNase I at 37°C for 10 min. After inhibition of the trypsin activity by addition of fetal calf serum, the dissociated cells were filtered through a 100-mesh (125 μm) screen and washed twice with PBS. For the neuron culture, the cells were seeded onto the poly-L-lysine-coated wells of 24-well plates at a density of 5×10^5 cells/well or 75-cm^2 culture flasks at a density of 5×10^7 cells/bottle in minimum essential medium containing 2% fetal bovine serum. The neurons were cultured for 5 days at 37°C in 5% CO_2. For the astrocyte culture, the cells were seeded onto 75-cm^2 flasks at a density of 2.0×10^7 cells/bottle in Dulbecco's modified essential medium containing 10% fetal bovine serum and cultured at 37°C in 5% CO_2 with a change of medium twice a week. After 1 week, nonastrocytes were removed by shaking (McCarthy and de Vellis, 1980). The resultant astrocytic monolayer was removed and dissociated by trypsin treatment. The cell suspension was seeded onto 24-well plates at a density of 2×10^5/well or onto 75-cm^2 culture flasks at a density of 2×10^7 cells. The astrocytes were cultured for further 5 days.

The purity of the neurons and the astrocytes in each primary culture was approximately 97% and over 98%, respectively, as determined by immunostaining with anti-neurofilament antibody (Dako A/S, Denmark) for neurons and with anti-GFAP antibody (Dako A/S) for astrocytes.

Immunofluorescent staining of neurons and astrocytes in primary culture with anti-SSEA-1 or anti-Fut9 mAb

Cultured cells on poly-L-lysine-coated cover glasses were fixed with 4% paraformaldehyde/PBS for 10 min at room temperature. After three washes with PBS, blocking was performed in 1% bovine serum albumin/PBS for 1 h at 4°C. The cells were incubated with the first antibodies, anti-SSEA-1 mAb or anti-Fut9 mAb, overnight at 4°C. They were washed with PBS three times and then incubated with the secondary antibodies, FITC-conjugated goat IgG fraction anti-mouse IgM (Cappel, ICN, OH) for

anti-SSEA-1 mAb or FITC-conjugated sheep IgG fraction anti-mouse IgG (Cappel, ICN) for anti-Fut9 mAb. For the staining of Fut9, which is intracellulary localized in the Golgi apparatus, all solutions for treatment contained 0.05% Triton X-100.

Immunohistochemical staining of P7 mouse brain

Brain tissues of P7 mice were fixed in 4% paraformaldehyde/PBS overnight at 4°C. After their equilibration in 20% sucrose/PBS, they were embedded in Tissue Mount (Chiba Medical, Chiba, Japan). Fresh frozen sections (6 μm thick) were washed in PBS at room temperature, treated with 0.3% (v/v) H_2O_2 in PBS for 15 min for the blocking of endogenous peroxidase, and then washed three times in PBS. An Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) was used prior to 30-min incubation with 0.5% normal swine serum (Dako A/S) and 0.1% NaN_3 in PBS at room temperature. The sections were incubated overnight at 4°C with each primary mAb, anti-Fut9 mAb, KM2681 (mouse IgG1), or anti-SSEA-1 mAb (mouse IgM). After three washes with PBS, binding was visualized using the streptavidin-biotin technique (Vector M.O.M. Immunodetection kit, Vector Laboratories), and nuclei were counterstained with 5% methyl green stain solution, pH 4.0 (Muto Pure Chemicals, Tokyo). The double staining with anti-Fut9 mAb and anti-SSEA-1 mAb was performed by using FITC-conjugated sheep IgG fraction anti-mouse IgG (Cappel, ICN) and rhodamine-labeled affinity purified antibody to mouse IgM (Kirkegaard & Perry Laboratories, MD), respectively. Confocal images were detected with the laser scanning microscope LSM510 (Zeiss, Germany).

Acknowledgments

We thank R. Watanabe and H. Fukumitsu (Division of Immunoscience, Institute of Life Science, Soka University) for helpful suggestions and discussions. We also thank T. Inoue and K. Hayashida (Animal House, Institute of Life Science, Soka University) for technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research C. No.12680620 from the Ministry of Education, Science, Sports, and Culture of Japan.

Abbreviations

2AB, 2-aminobenzamide; CNS, central nervous system; FITC, fluorescein isothiocyanate; Fut or FUT, fucosyltransferase; GAPDH, glyceraldehyde phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HPLC, high-pressure liquid chromatography; Le^x, Lewis X; 2LN, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc; 3LN, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc; LNnT, lacto-*N*-neotetraose; mAb, monoclonal antibody; nLc_4, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer; nLc_6, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-1′Cer; ORF, open reading frame; PBS, phosphate buffered saline; RT-PCR, reverse transcription polymerase chain reaction; SSEA-1, stage-specific embryonic antigen-1; TLC, thin-layer chromatography.

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