J. Biochem. 123, 1164-1168 (1998)

Development-Dependent Expression of Complex-Type Sugar Chains Specific to Mouse Brain

Shin-ichi Nakakita,* Shunji Natsuka,* Kazuhiro Ikenaka,† and Sumihiro Hase*,[1]

*Department of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 560; †National Institute for Physiological Sciences, Okazaki National Research Institutes, Okazaki, Aichi 444

Received for publication, January 13, 1998

We previously detected a fucosylagalactobiantenna with a bisecting GlcNAc residue (BA-2) and one lacking the GlcNAc residue linked to the Manα1-3 residue of BA-2 (BA-1), which were enriched specifically in mouse brain [Shimizu, H., Ochiai, K., Ikenaka, K., Mikoshiba, K., and Hase, S. (1993) *J. Biochem.* 114, 334-338]. Pyridylamino sugar chains were prepared from mouse brains of various ages, and BA-1 and BA-2 were quantified after separation by HPLC. In cerebrum, BA-1 was scarcely expressed in newborn brain but gradually increased in amount during development, while expression of BA-2 reached a maximum 1 week after birth followed by a rapid decrease; in adult mice, the amount of BA-1 was almost the same as that of BA-2. In cerebellum, expression of BA-1 was lower than that of BA-2 at all stages. Glycoproteins with the BA-1 and BA-2 structures were enriched in the membrane fraction, and the glycoproteins solubilized were purified by lectin-affinity chromatography and gel filtration. The results indicated that BA-1 and BA-2 occurred in glycoproteins of more than 20 kDa in cerebellum, but most BA-1 and BA-2 were found in a 80-200 kDa fraction in cerebrum. These results show that the two brain-specific sugar chains are developmentally regulated and linked to the membrane-associated glycoproteins of subcellular organellas.

Key words: brain-specific, development dependent, membrane glycoprotein, pyridylamination, sugar chain.

We have detected two brain-specific sugar chains (BA-1 and BA-2) in mouse neural tissues by the differential display of pyridylaminated glycans using HPLC, and determined their chemical structures (*1*): GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc (BA-1) and GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc (BA-2). The BA-2 structure has been reported as a major component of glycoproteins isolated from brain tissues of different mammalian species: human β trace protein from cerebrospinal fluid (*2*), human asialotransferrin from cerebrospinal fluid (*3*), and prion protein from scrapie hamster (*4*). These two sugar chains specific to brain contain a unique structure in that they terminate with 2 to 3 GlcNAc residues; these GlcNAc residues are covered with galactose in most *N*-glycans reported.

We report here on the characteristics of the two brain-specific sugar chains, their expression during the development of the mouse, and an analysis of the glycoproteins they are linked to, as a step towards an understanding the functions of these sugar chains.

MATERIALS AND METHODS

Materials—Cerebrum and cerebellum were prepared from ICR mice. TSK-gel HW-40F, TSK-gel Amide-80, and TSK-gel G 3000 SW XL were purchased from Tosoh (Tokyo), YMC-Gel Sil S-5 from Yamamura Kagaku (Kyoto), a Mono Q 5/5 HR column from Pharmacia (Uppsala, Sweden), Cosmosil 5C18-P from Nacalai Tesque (Kyoto), LCA-agarose from Seikagaku Kogyo (Tokyo), BCA protein assay reagent and Micro BCA protein assay reagent kits from Pierce (Rockford, IL), and molecular weight markers for SDS-PAGE from Amersham International (Buckingamshire, UK).

Preparation of PA-Sugar Chains—A lyophilized sample (1-2 mg) was hydrazinolyzed (100°C, 10 h) followed by *N*-acetylation as previously reported (*5*). The reducing ends of the liberated sugar chains were pyridylaminated with a Palstation model 1000 (Takara Biomedicals, Kyoto) under the conditions reported previously (*6*). Excess reagents were removed by gel filtration on a TSK-gel HW-40F column (1.5×20 cm) equilibrated with a 10 mM ammonium acetate buffer, pH 6.0.

High Performance Liquid Chromatography—A Nanospace SI-1 liquid chromatograph (Shiseido, Tokyo) was used. Reversed-phase HPLC was done on a Cosmosil 5C18-P column (0.15×25 cm) with two eluents, A and B; Eluent A was 20 mM ammonium acetate buffer, pH 4.0, and Eluent B 20 mM ammonium acetate buffer, pH 4.0,

[1] To whom correspondence should be addressed. Fax: +81-6-850-5383

Abbreviations: PA, pyridylamino; DOC, deoxycholic acid; LCA, *Lens culinaris* agglutinin; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; Fuc, L-fucose.

containing 0.5% 1-butanol. The column was equilibrated with 15% Eluent B. After injection of a sample, the proportion of Eluent B was linearly changed from 15 to 85% in 90 min at a flow rate of 0.15 ml/min at 25°C. Separation of PA-sugar chains on an anion-exchange column (Mono Q5/5 HR) was performed as reported elsewhere (*7*). Size-fractionation HPLC was carried out on a TSK-gel Amide-80 column (0.46×7.5 cm) with a guard column of YMC-Gel Sil-5 (0.75×7.5 cm) to prevent damage to the Amide-80 column as reported elsewhere (*1*).

Subcellular Fractionation of Mouse Brain—The subcellular fractions were prepared according to Jahn *et al.* (*8*). Briefly, cerebrum (0.8 g) and cerebellum (0.2 g) obtained from two adult ICR mice were separately homogenized in a 9-fold volume of 0.32 M sucrose using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1,000×*g* for 10 min. The pellet (Pellet 1) was removed, and the supernatant was transferred into new tubes and centrifuged at 15,000×*g* for 20 min. The pellet (Pellet 2) was again removed, and the supernatant was transferred into new tubes and centrifuged at 105,000×*g* for 60 min. A final pellet (Pellet 3) and supernatant (Sup) were obtained. Pellets 1 to 3 were separately suspended in 7 ml of water.

Isolation of BA-1 and BA-2 Enriched Glycoproteins—Cerebrum (6 g) and cerebellum (2 g) obtained from 20 adult ICR mice were separately homogenized as described above. The membrane fraction (Pellets 2 and 3) was prepared from the homogenate and suspended in a 10-fold volume of cold acetone. The suspension was left on ice for 15 min, then centrifuged at 10,000×*g* for 10 min. The precipitates were added to 25 ml of 10 mM Tris-HCl buffer, pH 8.0, and the suspension was centrifuged at 100,000×*g* for 30 min. The precipitates were homogenized with 27 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 2% DOC using a Potter-Elvehjem homogenizer at 4°C. The solubilized membrane fraction was centrifuged at 100,000×*g* for 30 min. The precipitates were re-homogenized with 27 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 2% DOC, and the homogenates were centrifuged at 100,000×*g* for 30 min. The combined supernatant was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% DOC. The non-dialysable fraction was applied onto a LCA-agarose column (1.8×8.0 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% DOC, and the column was washed with the same buffer. Unadsorbed proteins were washed out with 400 ml of the buffer, and the bound proteins were eluted with 0.4 M methyl α-D-mannopyranoside in the same buffer. The LCA-agarose bound fraction was collected and concentrated with an Amicon YM-3 membrane. The solution was applied to TSK-gel G 3000 SW XL equilibrated with 0.1 M acetate buffer, pH 5.5, containing 2% SDS. The column was eluted with the same buffer.

Polyacrylamide Gel Electrophoresis—SDS-PAGE was carried out by the method of Weber and Osborn (*9*) using a 5–20% gel, and proteins in the gel were stained with Coomassie Brilliant Blue R-250. The proteins used as molecular weight standards were myosin (M_r 220,000), phosphorylase *b* (M_r 97,400), bovine serum albumin (M_r 66,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,300). Quantitation of PA-sugar chains was done by reversed-phase HPLC with PA-GlcNAc as a standard, and proteins were determined with a BCA protein assay kit or Micro BCA protein assay kit using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Quantitation of BA-1 and BA-2 in Neural Tissues—The amounts of BA-1 and BA-2 were measured respectively by quantitating the BA-1-PA and BA-2-PA peaks separated by HPLC of the PA-sugar chains. The developmental dependencies of the expression of BA-1 and BA-2 were analyzed using embryonic day 16 and postnatal week 0–16 brain. The amount of *N*-linked sugar chains relative to proteins was not dependent on age, and development-dependent *N*-linked sugar chains other than BA-1 and BA-2 were scarcely detected. BA-2 was present in the cerebrum in the embryonic stage, reached the maximum amount on postnatal day 7, and remained constant after postnatal day 21 (Fig. 1A). BA-1 was not detected in embryonic cerebrum. After birth, it gradually increased to the same level as BA-2 by day 56 (Fig. 1A). In cerebellum, BA-2 was present on postnatal day 7, reached the maximum amount on postnatal day 21, and remained constant after postnatal day 28 (Fig. 1B). BA-1 appeared on postnatal day 14 and thereafter remained constant in cerebellum; at week 16 its amount was only 25% of that of BA-2 (Fig. 1B). These results show that the expression of both BA-1 and BA-2 changes characteristi-

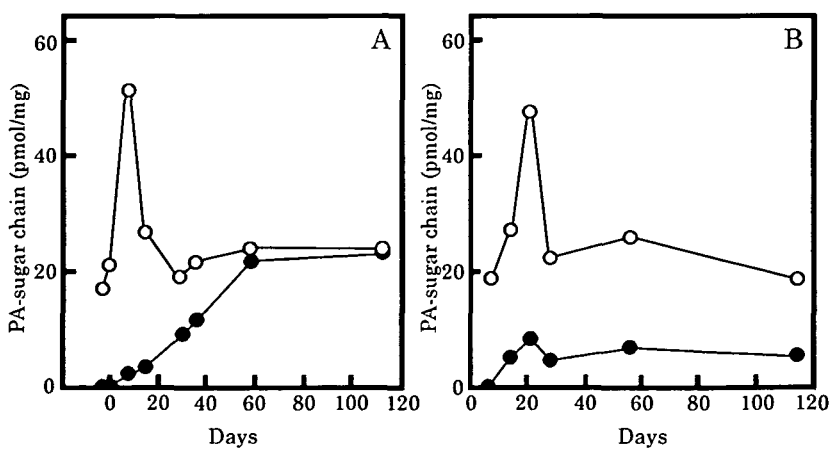

Fig. 1. **Amounts of BA-1 and BA-2 obtained from developing mouse brain.** BA-1 and BA-2 were quantitated as PA-sugar chains by HPLC as described in "MATERIALS AND METHODS." A, amounts of BA-1 (●) and BA-2 (○) from cerebrum; B, amounts of BA-1 (●) and BA-2 (○) from cerebellum.

cally with the development of the mouse brain. The rapid up-regulation of BA-2 in the cerebrum coincided with stage of synapse formation by neurons. It is possible that the presence of BA-2 existed in axons or synapses is associated with the extents of axons, the recognition of target cells of growth cones, and the formation of synapses.

Subcellular Localization of BA-1 and BA-2—The subcellular localization of the two sugar chains was analyzed by quantitating the BA-1 and BA-2 peaks separated by HPLC of PA-sugar chains obtained by hydrazinolysis of the subcellular fractions. Large amounts of BA-1 and BA-2, both in total and relative to protein, were detected in pellets 2 and 3 from cerebrum and cerebellum (Table I). These results indicate that BA-1 and BA-2 are mainly linked to membrane-associated glycoproteins of subcellular organellas.

Amounts of BA-1 and BA-2 Separated by Affinity and Gel Chromatography—As BA-1-PA and BA-2-PA bound to LCA (*10*), glycoproteins with BA-1 and BA-2 structures were purified from the solubilized membrane fractions by affinity chromatography on LCA-agarose (Fig. 2). More than 90% of BA-1 and BA-2 was found in the adsorbed fraction, which was further separated into four fractions, 1-4, by gel chromatography (Fig. 3). The positions on SDS-PAGE of proteins obtained from cerebrum were almost the same as those from cerebellum. The intensities of stained bands with CBB, however, were different between cerebrum and cerebellum. An 80 kDa protein was strongly stained in cerebrum, but weakly stained in cerebellum (Fig. 4, A-2 and B-2). The fact that the amounts of BA-1 and BA-2 relative to proteins in fraction 2 were almost the same in cerebrum and cerebellum (Fig. 5, B and D) suggests that BA-1 and BA-2 were scarcely expressed in 80 kDa protein. In contrast, 45 kDa protein of cerebellum was strongly stained, but that of cerebrum weakly stained. The amount of BA-2 relative to proteins in fraction 3 of cerebellum was larger than that in cerebrum. These results

TABLE I. **Intracellular localization of BA-1 and BA-2 in mouse brain.**

(A) Cerebrum

	Protein (mg)[a]	BA-1 (pmol)	BA-1/protein (pmol/mg)	BA-2 (pmol)	BA-2/protein (pmol/mg)
Pellet 1	6.8	140	20	190	28
Pellet 2	24	2,000	81	2,500	103
Pellet 3	15	1,750	120	2,100	140
Sup	12	300	26	138	12

(B) Cerebellum

	Protein (mg)[a]	BA-1 (pmol)	BA-1/protein (pmol/mg)	BA-2 (pmol)	BA-2/protein (pmol/mg)
Pellet 1	3.4	230	66	370	120
Pellet 2	8.6	600	69	1,500	170
Pellet 3	1.5	160	108	430	290
Sup	4.2	97	23	130	31

[a]The amount of protein was determined by BCA protein assay reagent with bovine serum albumin as a standard.

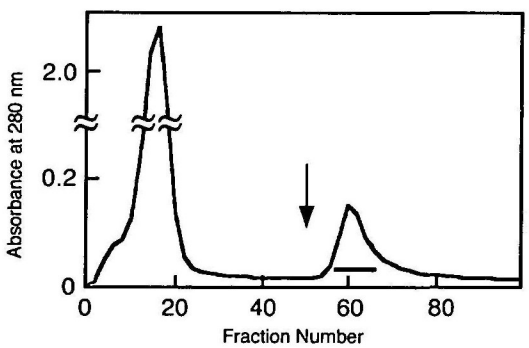

Fig. 2. **LCA-agarose affinity chromatography of DOC extract obtained from the cerebrum membrane fraction.** The arrow indicates the position where the elution buffer containing 0.4 M methyl α-D-mannoside was used as the eluent. The fraction indicated by the bar was collected. The flow rate was 5 ml/h, and 8-ml fractions were collected.

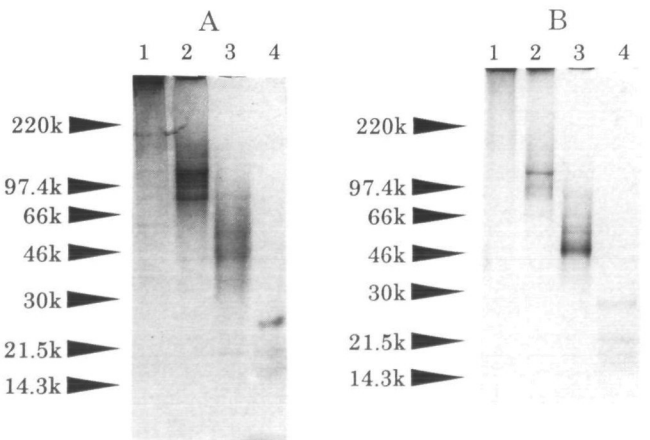

Fig. 4. **SDS-PAGE of Fractions 1-4.** Fractions 1-4 obtained in Fig. 3 were analyzed by SDS-PAGE under non-reducing conditions. Lanes: 1, Fraction 1; 2, Fraction 2; 3, Fraction 3; 4, Fraction 4. The proteins were stained with Coomassie Brilliant Blue R 250. A, cerebrum; B, cerebellum.

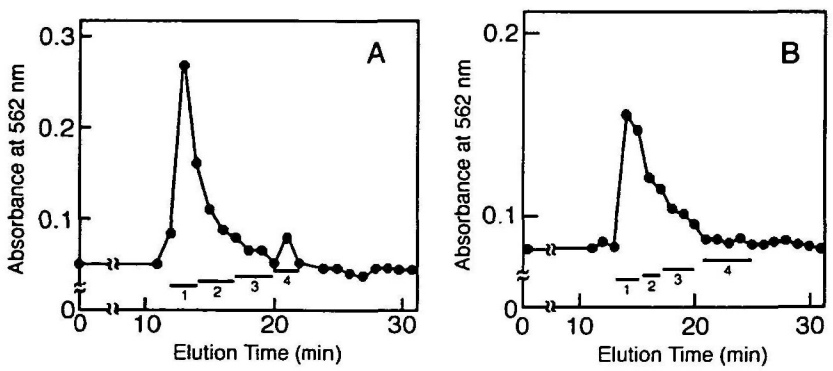

Fig. 3. **TSK-gel G 3000 SW chromatography of the fraction obtained in Fig. 2.** The fractions indicated by bars were collected. The flow rate was 0.5 ml/min, and 0.5-ml fractions were collected. Proteins were detected with a BCA protein assay kit. A, cerebrum; B, cerebellum.

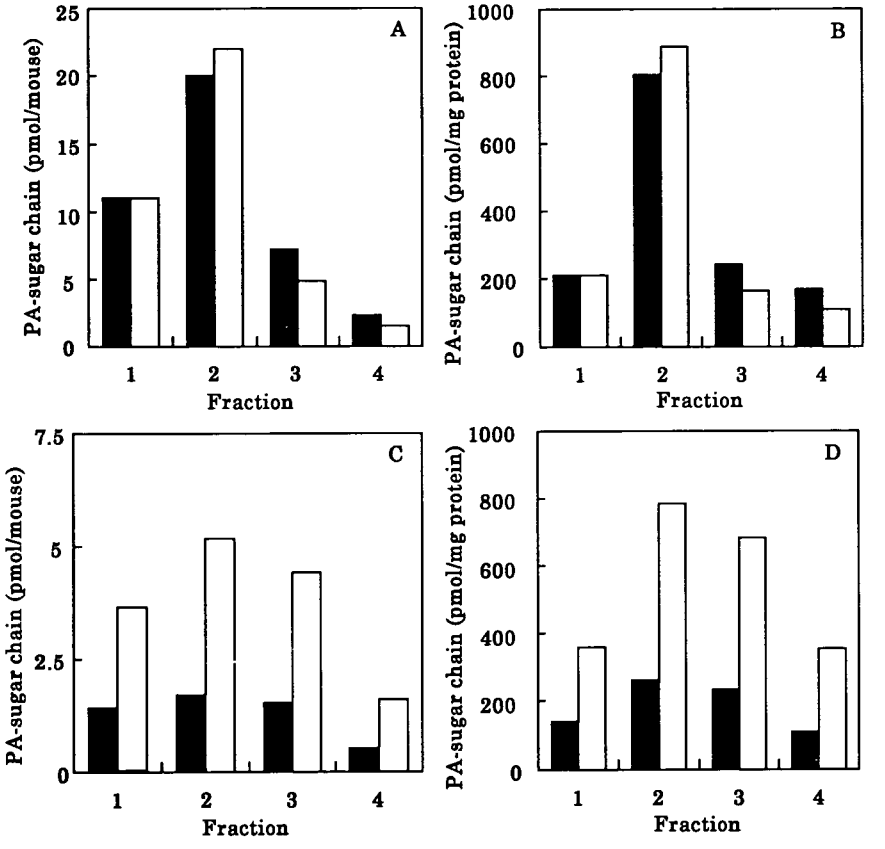

Fig. 5. **Total amounts and amounts relative to protein of BA-1 and BA-2 in Fractions 1-4.** BA-1 and BA-2 were quantitated by measuring the PA-sugar chains using HPLC as described in "MATERIALS AND METHODS." A, total amounts of BA-1 (black columns) and BA-2 (white columns) of 8-week mouse cerebrum; B, amounts relative to protein of BA-1 (black columns) and BA-2 (white columns) of 8-week mouse cerebrum; C, total amounts of BA-1 (black columns) and BA-2 (white columns) of 8-week mouse cerebellum; D, amounts relative to protein of BA-1 (black columns) and BA-2 (white columns) of 8-week mouse cerebellum. Loss during purification was not considered.

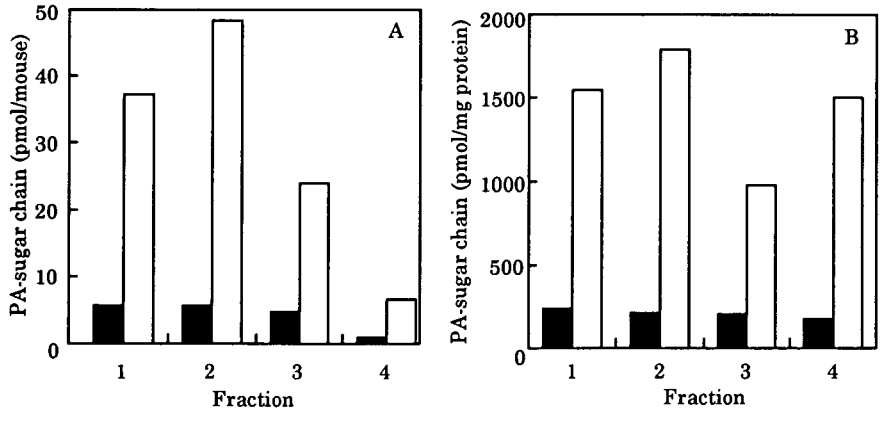

Fig. 6. **Amounts of BA-1 and BA-2 in Fractions 1-4 obtained from cerebrum of 1-week mouse.** BA-1 and BA-2 were quantitated as PA-sugar chains by HPLC as described in "MATERIALS AND METHODS." A, total amounts of BA-1 (black columns) and BA-2 (white columns); B, amounts relative to protein of BA-1 (black columns) and BA-2 (white columns). Loss during purification was not considered.

suggest that BA-2 was strongly expressed in 45 kDa protein. Thus the expressions of BA-1- and BA-2-associated glycoproteins were different between cerebrum and cerebellum.

The distributions of BA-1 and BA-2 in cerebrum on postnatal day 7, when BA-2 in cerebrum reached its maximum (Fig. 1A), were examined by conducting the same experiment as described above. Subcellular localization, elution pattern of glycoproteins on LCA-agarose and G3000SW, and CBB staining pattern of proteins of postnatal day 7 cerebrum were almost same as those of postnatal week 8 (data not shown). The amounts of BA-2 relative to protein were the same in fractions 1-4 of postnatal day 7 (Fig. 6). These results suggest that BA-2 was expressed in various glycoproteins at postnatal day 7, but mainly in glycoproteins of 200 to 80 kDa in adult mouse.

BA-1 lacks one GlcNAc residue linked to C-2 of the Manα1-3 residue. This GlcNAc residue is transferred to $Man_5GlcNAc_2$ by *N*-acetylglucosaminyltransferase I during the biosynthesis of *N*-linked sugar chains, and this is a necessary step for the further addition of Fuc and bisecting GlcNAc residues. These results suggest that BA-2 was hydrolyzed to BA-1 by a β-*N*-acetylglucosaminidase present in the cerebrum.

The present study shows that the two brain-specific sugar chains are developmentally regulated and linked to the glycoproteins located in the membranes.

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