J. Biochem. 125, 537-540 (1999)

Conversion of Brain-Specific Complex Type Sugar Chains by *N*-Acetyl-β-D-Hexosaminidase B[1]

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Received October 19, 1998; accepted December 7, 1998

The *N*-linked sugar chains, 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), were recently found to be linked to membrane proteins of mouse brain in a development-dependent manner [S. Nakakita, S. Natsuka, K. Ikenaka, and S. Hase, *J. Biochem.* 123, 1164-1168 (1998)]. The GlcNAc residue linked to the Manα1-3 branch of BA-2 is lacking in BA-1 and the removal of this GlcNAc residue is not part of the usual biosynthetic pathway for *N*-linked sugar chains, suggesting the existence of an *N*-acetyl-β-D-hexosaminidase. Using pyridylaminated BA-2 (BA-2-PA) as a substrate the activity of this enzyme was found in all four subcellular fractions obtained. The activity was much greater in the cerebrum than in the cerebellum. To further identify the *N*-acetyl-β-D-hexosaminidase, BA-1 and BA-2 in brain tissues of *Hex* gene-disrupted mutant mice were detected and quantified. PA-sugar chains were liberated from the cerebrum and cerebellum of the mutant mice by hydrazinolysis-*N*-acetylation followed by pyridylamination. PA-sugar chains were separated by anion-exchange HPLC, size-fractionation, and reversed-phase HPLC. Each peak was quantified by measuring the peaks at the elution positions of authentic BA-1-PA and BA-2-PA. BA-2-PA was detected in all the PA-sugar chain fractions prepared from *Hexa*, *Hexb*, and both *Hexa* and *Hexb* (double knockout) gene-disrupted mice, but BA-1 was not found in the fractions from *Hexb* gene-disrupted and double knockout mice. These results indicate that *N*-acetyl-β-D-hexosaminidase B encoded by the *Hexb* gene hydrolyzed BA-2 to BA-1.

Key words: brain, hexosaminidase, sugar chains.

We previously reported two *N*-linked sugar chains specifically found in the brain on comparison of the HPLC profiles of PA-sugar chains. Their structures were determined to be 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) (*1*). These sugar chain structures have been reported to occur mostly in brain-related glycoproteins, including human β-trace protein (*2*), human asialotransferrin from cerebrospinal fluid (*3*), prion protein from scrapie hamster (*4*), and rat brain (*5*). Human immunoglobulin G contains the BA-2 structure as a minor component (*6, 7*). BA-1 and BA-2 are developmentally regulated and linked to membrane-associated glycoproteins. They are thought to be involved in the extension of axons, recognition of target cells of growth cones, and formation of synapses (*8*). In a recent study (*8*), BA-1 was found to be absent in newborn mice. In the cerebrum, it gradually increased with age to reach the level of BA-2; however, in the cerebellum BA-1 remained up to day 3 and then gradually increased, but only to a low level. The specific structural feature of BA-1 is that the GlcNAc residue linked to the Manα1-3 branch is absent. This GlcNAc residue is first transferred to the Man5GlcNAc2 structure by GlcNAc transferase I prior to the addition of a bisecting GlcNAc residue and the Fucα1-6 residue linked to the reducing-end GlcNAc residue (*9*). Therefore, the absence of the GlcNAc residue in BA-1 suggests the action of an *N*-acetyl-β-D-hexosaminidase on BA-2 after the biosynthesis of glycans. Several *N*-acetyl-β-D-hexosaminidases have been reported to be present in cells, among which lysosomal *N*-acetyl-β-D-hexosaminidases A, B, and S have received most attention. Their gene structures have been determined, and mutant mice in which the *Hexa* and/or *Hexb* gene is disrupted have been

[1] This study was supported in part by a grant under the Research for the Future Program of the Japan Society for the Promotion of Science (to S.H.), and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No. 09770158, to S.Y.).

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Abbreviations: BA-1, GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; BA-2, GlcNAcβ1-2Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; HPLC, high-performance liquid chromatography; PA-, pyridylamino.

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produced (*10, 11*). To determine which *N*-acetyl-β-D-hexosaminidase is responsible for the processing of BA-1, in the present study we quantified BA-1 and BA-2 in brain tissues of mutant mice. Our results showed that *N*-acetyl-β-D-hexosaminidase B hydrolyzed BA-2 to BA-1.

MATERIALS AND METHODS

Materials—*Hexa*, *Hexb*, and both *Hexa* and *Hexb* gene-disrupted (double knockout) mice were obtained from Dr. R.L. Proia (*12*). Standard BA-1-PA and BA-2-PA were prepared with the hen egg yolk antibody, IgY, as described previously (*13*). *p*-Nitrophenyl β-D-glucosaminide was purchased from Sigma (St. Louis, MO). Cosmosil 5C18-P columns (0.6×25 cm and 0.15×25 cm) were purchased from Nacalai Tesque (Kyoto), Toyopearl HW-40F from Tosoh (Tokyo), a Shodex Asahipack NH2P-50 column (0.46×5 cm) from Showa Denko (Tokyo), and a Mono Q HR 5/5 column (0.5×5 cm) from Pharmacia (Uppsala).

Subcellular Fractionation of Mouse Brain—Subcellular fractions were prepared according to Jahn *et al.* (*14*). Briefly, cerebra (0.8 g) and cerebella (0.2 g) obtained from two adult ICR mice or mutant mice were separately homogenized in a 9-fold volume of 0.005 M Tris-HCl buffer, pH 7.4, containing 0.32 M sucrose using a Potter-Elvehjem homogenizer. Each homogenate was centrifuged at 1,000×*g* for 10 min. The pellet (P1) was removed, and the supernatant was transferred to a new tube and then centrifuged at 11,500×*g* for 20 min. The pellet (P2) was again discarded, and the supernatant was transferred to a new tube and then centrifuged at 105,000×*g* for 60 min. A final pellet (P3) and supernatant (S) were obtained.

Analysis of Enzyme Activity—*N*-Acetyl-β-D-hexosaminidase activity was assayed using 0.08 mM BA-2-PA and tissue corresponding to 50 μg protein [assayed using a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard] in 75 μl of 0.05 M sodium 3,3-dimethylglutarate buffer, pH 5.0, for 20 h at 37°C. The enzyme activity increased linearly up to 24 h. The enzymatic reaction was terminated by adding 75 μl of 2 M acetic acid, and then the products were analyzed by reversed-phase HPLC on a Cosmosil 5C18-P (0.6×25 cm) column as described below. The enzyme activity was also assayed using *p*-nitrophenyl β-*N*-acetylglucosaminide as a substrate. The enzyme solution (20 μg protein) and 5.0 mM *p*-nitrophenyl β-*N*-acetylglucosaminide in 100 μl of sodium 3,3-dimethylglutarate buffer, pH 5.0, were incubated at 37°C for 30 min. The enzymatic reaction was terminated by adding 900 μl of 0.5 M Na_2CO_3. The *p*-nitrophenol released was quantified by measuring the absorbance at 400 nm.

Preparation of PA-Sugar Chains from Mouse Brain—One milligram of lyophilized mouse brain was hydrazinolyzed (100°C, 10 h), followed by *N*-acetylation. The sugar chains liberated were pyridylaminated as described previously (*8, 15, 16*). The PA-sugar chains were separated from excess reagents by gel filtration through an HW-40F column (1.5×40 cm; Tosoh, Tokyo) equilibrated with 10 mM ammonium acetate buffer, pH 6.0, and the fraction between the elution volumes of 18 and 48 ml was collected.

High-Performance Liquid Chromatography—Elution was monitored as to the fluorescence, the excitation and emission wavelengths being 320 and 400 nm, respectively.

Reversed-phased HPLC for determination of the enzyme activity was performed on a Cosmosil 5C18-P column (0.6×25 cm) at the flow rate of 1.5 ml/min at 25°C. The eluent used was 0.1 M ammonium acetate buffer, pH 4.0, containing 0.18% 1-butanol. Reversed-phase HPLC for detection of BA-1 and BA-2 was performed on a Cosmosil 5C18-P column (0.15×25 cm) at the flow rate of 150 μl/min at 25°C. The eluents used were 20 mM ammonium acetate buffer, pH 4.0 (Eluent A), and 20 mM ammonium acetate buffer, pH 4.0, containing 0.5% 1-butanol (Eluent B). The column was equilibrated with 15% Eluent B. After injecting a sample, the proportion of Eluent B was increased linearly to 80% in 90 min. Size-fractionation HPLC was carried out on a Shodex Asahipak NH2P-50 column at 25°C at the flow rate of 0.6 ml/min. Two eluents, C and D, were used. Eluent C was acetonitrile:water:acetic acid (200:800:3, v/v/v) titrated to pH 7.0 with aqueous ammonia, and Eluent D was acetonitrile:water:acetic acid (930:70:3, v/v/v) titrated to pH 7.0 with aqueous ammonia. The column was equilibrated with Eluent D:Eluent C (32:1, v/v). After injecting a sample, linear gradient elution was performed to Eluent D:Eluent C at the ratio of 2:1 (v/v) in 1 min and then 3:7 (v/v) in 34 min. Mono Q HPLC was performed on a Mono Q HR 5/5 column at 25°C at the flow

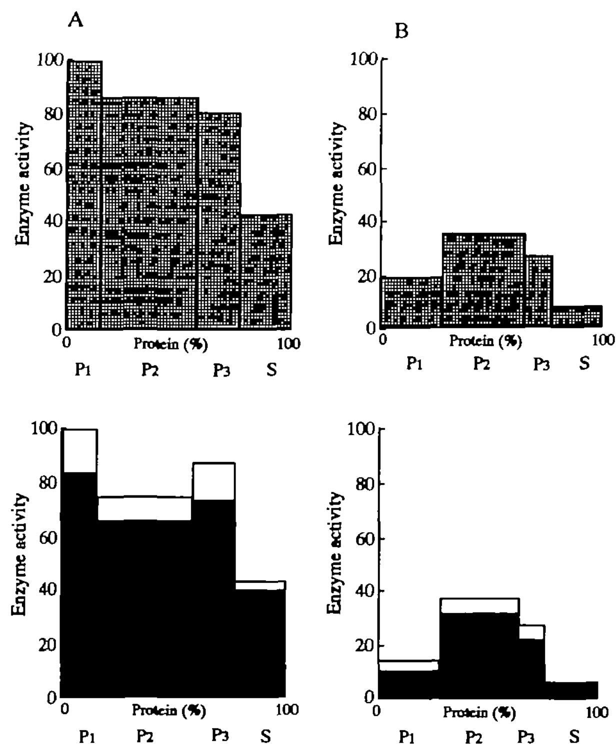

Fig. 1. **Distribution of *N*-acetyl-β-D-hexosaminidase activity in subcellular fractions of the cerebrum (A) and cerebellum (B) of normal mice.** Cells were homogenized and fractionated into fractions P1, P2, P3, and S, as described under "MATERIALS AND METHODS." The substrates used were *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (shaded columns) and BA-2-PA (black and white columns). Black columns indicate that the product was BA-1-PA and white columns that it was Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Man-α1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-PA. Enzyme activities are indicated per mg protein on the ordinate, the activity of the P1 fraction of the cerebrum being taken as 100.

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rate of 1.0 ml/min using two eluents: Eluent A was water titrated to pH 9.0 with aqueous ammonia, and Eluent B was a 0.5 M ammonium acetate solution, pH 9.0. The column was equilibrated with Eluent A. After injecting a sample, Eluent B was increased to 10% in 3 min, to 40% in 14 min, and then to 100% in 5 min.

RESULTS AND DISCUSSION

N-Acetyl-β-D-Hexosaminidase Activity in Subcellular Fractions—The enzyme activities in subcellular fractions obtained from ICR mice were analyzed using two substrates, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and BA-2-PA (Fig. 1). Higher *N*-acetyl-β-D-hexosaminidase activity toward both substrates was observed in the cerebrum than in the cerebellum, and the results were comparable with the reported data (*17*). The enzyme activity was found in all four fractions. The GlcNAc residue linked to the Manα1-3 branch was hydrolyzed faster than that linked to the Manα1-6 branch. The ratio of BA-1-PA to its isomer, Manα1-6(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-3)Manα1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-PA, differed among the fractions, indicating that more than one enzyme which acts on BA-2-PA and *p*-nitrophenyl *N*-acetylglucosaminide exists. To identify the enzyme that converts BA2 to BA1, the sugar chains of the brains of *Hex* gene-disrupted mice were examined.

Quantification of BA-1 and BA-2 in the Brains of Normal and Hex Gene-Disrupted Mouse Brain—Sugar chains liberated by the hydrazinolysis-*N*-acetylation method from lyophylized cerebrum and cerebellum were pyridylaminated. The PA-sugar chain fraction obtained on gel filtration on Toyopearl HW-40F was further separated by Mono-Q HPLC, and the pass-through fraction (neutral fraction), which contained BA-1-PA and BA-2-PA, was further purified by size-fractionation HPLC. The fractions at the elution positions of BA-1-PA and BA-2-PA were collected as already reported (*8*) (data not shown), and then each fraction was analyzed on a reversed-phase HPLC column (0.15×25 cm) (Fig. 2, A and B). The peak at the position of BA-2-PA was detected for all the PA-sugar chain fractions obtained from both the cerebrum and cerebellum. However, the peak at the elution position of BA-1 did not appear for the fractions purified from the cerebrum and cerebellum of *Hexb* gene-disrupted mice, whereas it was detected for *Hexa* gene-disrupted mice. The very small peaks in Fig. 2, A3 and B3, differed from BA-1-PA as they were not eluted at the exact position of BA-1-PA. These results indicate that the *Hexb* gene product hydrolyzed BA-2 to BA-1. *N*-Acetyl-β-D-hexosaminidase A consists of two non-identical subunits, the *Hexa* gene product (α-subunit) and the *Hexb* gene product (β-subunit), while *N*-acetyl-β-D-hexosaminidase S has two α-subunits, and *N*-acetyl-β-D-hexosaminidase B two β-subunits (*18*). Therefore, the *Hexb* gene product, *N*-acetyl-β-D-hexosaminidase B, was responsible for the hydrolysis of BA-2 in the cells, and *N*-acetyl-β-D-hexosaminidase S was not. However, the contribution of *N*-acetyl-β-D-hexosaminidase A remains unclear.

N-Acetyl-β-D-hexosaminidase is located in lysosomes, and it is considered that there is no chance of cell-membrane glycoproteins with the BA-1 and BA-2 structures being hydrolyzed by an enzyme that resides in lysosomes. However, a recent report has indicated that the mannose residues of cell-membrane glycoproteins are hydrolyzed after they leave the Golgi apparatus (*19*). This may be the case also for the glycoprotein with the BA-2 structure. The oligosaccharides with GlcNAc at their non-reducing termini

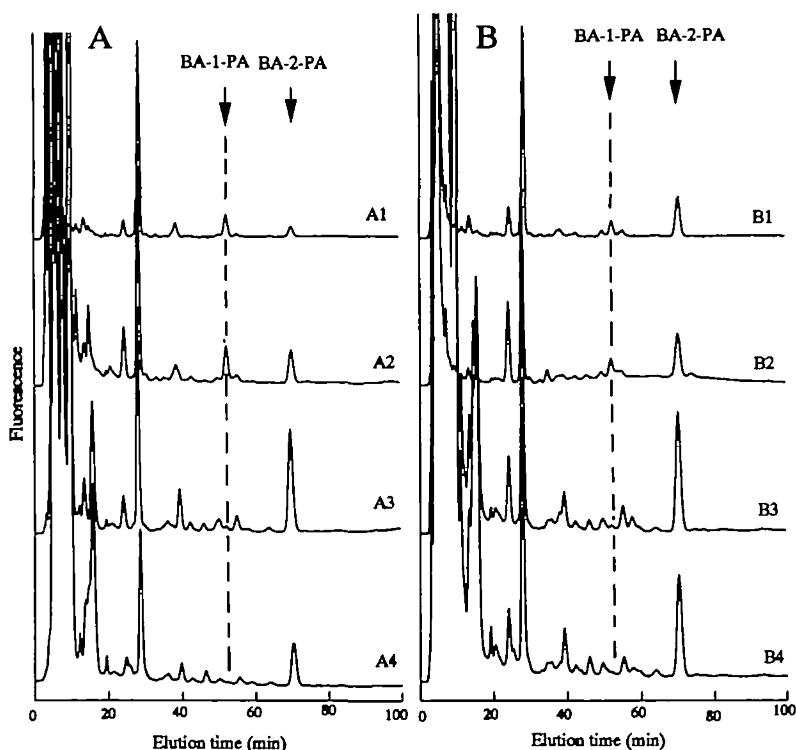

Fig. 2. **Reversed-phase HPLC of the BA-1-PA and BA-2-PA fractions purified by size-fractionation HPLC from the cerebrum (A) and cerebellum (B).** One-tenth of the solution obtained was injected. HPLC profiles of PA-sugar chains were obtained for: 1, ICR mice; 2, *Hexa* gene-disrupted mice; 3, *Hexb* gene-disrupted mice; 4, double knockout mice.

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derived from glycoproteins are ubiquitous storage compounds in Sandhoff diseases with hexosaminidase B deficiency. These oligosaccharides are accumulated and excreted in the urine (*20*). These results also support the results obtained in the present study.

The authors are grateful to Dr. R.L. Proia for supplying the mutant mice, and Dr. K.L. Menon for critical reading of the manuscript.

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