Purification and Characterization of a Glucuronyltransferase Involved in the Biosynthesis of the HNK-1 Epitope on Glycoproteins from Rat Brain*

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The glucuronyltransferase involved in the biosynthesis of the HNK-1 epitope on glycoproteins was purified to an apparent homogeneity from the Nonidet P-40 extract of 2-week postnatal rat forebrain by sequential chromatographies on CM-Sepharose CL-6B, UDP-GlcA-Sepharose 4B, asialo-orosomucoid-Sepharose 4B, Matrix gel Blue A, Mono Q, HiTrap chelating, and HiTrap heparin columns. The purified enzyme migrated as a 45-kDa protein upon SDS-polyacrylamide gel electrophoresis under reducing conditions, but eluted as a 90-kDa protein upon Superose gel filtration in the presence of Nonidet P-40, suggesting that the enzyme forms homodimers under non-denatured conditions. The enzyme transferred glucuronic acid to various glycoprotein acceptors bearing terminal N-acetyllactosamine structure such as asialo-orosomucoid, asialo-fetuin, and asialo-neural cell adhesion molecule, whereas little activity was detected to paragloboside, a precursor glycolipid of the HNK-1 epitope on glycolipids. These results suggested that the enzyme is specifically involved with the biosynthesis of the HNK-1 epitope on glycoproteins. Sphingomyelin was specifically required for expression of the enzyme activity. Stearyl-sphingomyelin (18:0) was the most effective, followed by palmitoyl-sphingomyelin (16:0) and lignoceroyl-sphingomyelin (24:0). Interestingly, activity was demonstrated only for sphingomyelin with a saturated fatty acid, i.e. not for that with an unsaturated fatty acid, regardless of the length of the acyl group.

Various cell surface carbohydrate moieties are thought to be involved in cell-to-cell interactions (1, 2). The HNK-1 carbohydrate epitope, which is recognized by HNK-1 monoclonal antibody, is found on many neural cell adhesion molecules, such as neural cell adhesion molecule (NCAM)3 (3), myelin-associated glycoproteins (4), L1 (3), transiently expressed axonal glycoprotein-1 (5), and P0 (6), and some proteoglycans (7). Expression of the HNK-1 carbohydrate epitope is spatially and temporally regulated during development, and its highest expression is seen at the stages where the neural networks are constructed in the central and peripheral nervous systems (8–10). The HNK-1 epitope is presumed to be involved in cell-to-cell interactions such as cell adhesion (11), migration (12), and neurite extension (13).

The epitope is expressed not only on glycoproteins but also on glycolipids. The structures of the HNK-1 reactive glycolipids are: SGGL-1 (HSO₃-GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer) and SGGL-2 (HSO₃-GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-Cer) (14, 15). The inner unit of their glycolipids, Galβ1-4GlcNAcβ1, is commonly found in mammalian glycoproteins and glycolipids, and the unique feature of this epitope is the terminal sulfo-3-glucuronyl group. This structure was shown to be essential not only for immuno-reactivity with HNK-1 monoclonal antibodies (16) but also for their functions (17). Therefore, in order to study the functions of the HNK-1 carbohydrate epitope during construction of the central and peripheral nervous systems, it is important to characterize the glucuronyltransferase that transfers glucuronic acid from uridine 5'-diphosphoglucuronic acid to the terminal N-acetyllactosamine structure of glycoproteins and glycolipids.

In our previous study, it was demonstrated that there are two types of glucuronyltransferases associated with the biosynthesis of the HNK-1 carbohydrate epitope in rat brain, one for glycolipid acceptors (18) and the other for glycoprotein acceptors (19). Similar glucuronyltransferase activities were found in chick (20) and rat (21) brains with paragloboside as an acceptor. In this study, a glucuronyltransferase specific for glycoprotein acceptors (GlcAT-P) was purified to apparent homogeneity from postnatal 2-week rat forebrains by means of various column chromatographies. The enzyme is a 45-kDa protein, which requires sphingomyelin (SM) for the expression of its transferase activity.

**EXPERIMENTAL PROCEDURES**

**Materials—**UDP-[1⁴C]GlcA (10.2 GBq/mmol) was purchased from ICN Radiochemicals. UDP-GlcA, UDP-GlcNAc, ATP, GlcA, Nonidet P-40, and benzimidine were from Nakalai Tesque Inc. (Kyoto, Japan). MES was from Dojindo (Kumamoto, Japan). UDP, aprotinin, and pepsatatin A were purchased from Sigma. Heparin and phenylmethylsulfonyl fluoride is from Wako Chemicals (Osaka, Japan). Heparin sulfate, hyaluronic acid, chondroitin, and chondroitetrascerylceramide (nLc-Cer) were from Seikagaku Corp. (Tokyo, Japan). N-Acetyllactosamine was provided by Yaizu Suisan Kagaku Kogyo Inc.

The abbreviations used are: NCAM, neural cell adhesion molecule; ASOR, asialo-orosomucoid; GlcAT, glucuronyltransferase; GlcAT-P, glycoprotein-specific GlcAT; MES, 2-(N-morpholino)ethanesulfonic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylinerine; PS, phosphatidylethanolamine; SM, sphingomyelin; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.
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(Yaizu, Japan). Orosomucoid was provided by Dr. M. Wickerhauser of the American Red Cross Research Center (Bethesda, MD). ASOR (asialo-orosomucoid) was prepared by hydrolysis of orosomucoid with 0.05 M H2SO4 for 1 h at 80 °C (22). Dye Matrex Blue A-agarose was obtained from Amicon (Daverformed, MA). CM-Sepharose CL-6B, EAH- Sepharose, CNBr-activated Sepharose 4B, and Mono Q, HiTrap chelating, and HiTrap heparin columns were from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). An anti-NCAM monoclonal antibody (AF11) (23) was kindly provided by Dr. Katsuhiro Ono (Shimane Medical College, Japan). Postnatal 2-week Wistar rats were purchased from Oriental Bio-service (Kyoto, Japan). The Wistar rats were acclimatized with diethyl ether and then sacrificed. Their brains were removed and immediately frozen on dry ice. The frozen rat brains were stored at −80 °C.

Preparation of Affinity Resin—ASOR-Sepharose 4B and anti-NCAM antibody-conjugated Sepharose 4B were performed by coupling ASOR (300 mg) to CNBr-activated Sepharose 4B (30 ml) and AfI1 antibody (5 mg) to CNBr-Sepharose 4B (5 ml), respectively, according to the procedure described previously (19). UDP-GlcA-Sepharose 4B was prepared by coupling UDP-GlcA (1 g) to EAH-Sepharose 4B (100 ml) according to the procedure described by Anttinen and Kivirikko (24).

Gluconuronyltransferase Assay—Gluconuronyltransferase activity toward glycoprotein acceptors was measured essentially as described previously (19) with slight modification. Incubation was carried out at 37 °C for 18 h in 200 µl of a mixture containing 20 ng of enzyme (prepared in 0.5 M NaCl, 0.7 µg/ml pepstatin A, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.7 µg/ml peptatin A, 10 µg/ml benzamidine, 0.1 mg/ml FMSF. Extracting buffer consisted of the homogenizing buffer containing 0.5% (v/v) Nonidet P-40, 20 mM MES buffer, pH 6.5, and 0.2% (v/v) Nonidet P-40 extract of rat forebrain, which had been treated at 100 °C for 3 min, in a final volume of 50 µl. After incubation, the assay mixture was spotted onto a 2.5-cm Whatman No. 1 disc and the radioactivity of [14C]GlcA-ASOR on the discs was counted with a liquid scintillation counter (Beckman LS-6000). Protein was quantitated with a Micro-BCA protein assay kit (Pierce) unless otherwise stated. Bovine serum albumin was used as a standard.

Purification of a Glucuronidase for Glycoprotein Acceptors—Homogenizing buffer consisted of 20 mM MES buffer, pH 6.5, 0.32 M sucrose, 1 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 1 µg/ml aprotinin, 0.7 µg/ml pepstatin A, 10 µg/ml benzamidine, 0.1 mg/ml FMSF. Extracting buffer consisted of the homogenizing buffer containing 0.5% (v/v) Nonidet P-40 instead of sucrose. Buffer A consisted of 20 mM MES buffer, pH 6.5, 0.5% Nonidet P-40. Buffer B consisted of 20 mM MES buffer, pH 6.5, 0.5% Nonidet P-40, 1 mM EDTA. Buffer C consisted of 10 mM MES buffer, pH 6.5, 0.5% NaCl, 0.1% Nonidet P-40, 1 mM EDTA. Buffer D consisted of 10 mM MES buffer, pH 6.5, 0.1% Nonidet P-40, 20 mM NaCl, 1 mM EDTA. Buffer E consisted of 10 mM MES buffer, pH 6.5, 0.1% Nonidet P-40, 1 mM EDTA. Buffer F consisted of 20 mM Tris/HCl buffer, pH 8.0, 0.1% Nonidet P-40, 10 mM NaCl. Buffer I consisted of 20 mM Tris/HCl buffer, pH 8.0, 0.1% Nonidet P-40, 0.5 M NaCl. Buffer II consisted of 10 mM MES buffer, pH 6.5, 0.1% Nonidet P-40, 0.25 mM NaCl. Buffer G consisted of 10 mM MES buffer, pH 6.5, 0.1% Nonidet P-40, 0.1 mM NaCl. Buffer H consisted of 20 mM Tris/HCl buffer, pH 8.0, 0.1% Nonidet P-40, 10 mM NaCl. Buffer I consisted of 300 mM NaCl, 1 mM EDTA. Buffer J consisted of 300 mM NaCl, 1 mM EDTA. Buffer K consisted of 30 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA. Buffer L consisted of 30 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA. Buffer M consisted of 10 mM MES buffer, pH 6.5, 0.1% Nonidet P-40, 1 mM NaCl.

Preparation of Phospholipids from Rat Brains—Total lipids were extracted from lyophilized rat brains (8.4 g) twice with 200 ml of chloroform-methanol, 2:1 (v/v), and then once with 250 ml of chloroform-methanol, 1:1 (v/v). The chloroform-methanol extract was subjected to Folch’s partitioning (26). The lower phase (1.87 g) was charged onto a DEAE-Sephadex A-25 column (2 × 40 cm, acetate form; Pharmacia LKB Biotechnology) equilibrated with chloroform-methanol-water, 30:60:8 (v/v), and then the column was eluted successively with the same solvent (five bed volumes) and with 0.45 M ammonium acetate in methanol. The first peak which eluted before the phosphatidylcholine peak was applied to an Alumina column (1 × 20 cm, ICN Alumina N-Super I, ICN Biomedicals GmbH, Eschwege, Germany). The column was successively eluted with 200 ml each of chloroform, chloroform-methanol, 95:5 (v/v), and chloroform-methanol-water, 60:40:10 (v/v), and then chloroform-methanol-water, 40:60:10 (v/v). Phosphatidylcholine (PC, 23 mg) was recovered from the chloroform-methanol (95:5) eluate in an amount of 100 mg of PC, mixed with 20 µg of the chloroform-methanol (80:20) eluate, and a mixture of phosphatidylcholine and lyso-PE from the chloroform-methanol-water eluate. Separation of PE and lyso-PE was carried out by Iatrobeads column (1 × 60 cm, 6RS-8060; Iatron Laboratory, Tokyo, Japan) chromatography. The column was eluted with a solvent mixture of chloroform-methanol-water, 65:25:4 (v/v). The yields of PE and lyso-PE fractions were 14.9 and 7.0 mg, respectively. The phosphatidylserine (PS) and phosphatidylethanolamine (PE) fractions were pooled, deacetylated with 0.5 M KOH in methanol at 37 °C for 6 h. The yield of the deacetylated neutral glycolipid fraction containing ceramide mono-, di-, and trisaccharides was 2.7 mg. The deacetylated SM fraction was applied to a QAE-Sepharose A-25 column (2 × 40 cm) form; Pharmacia LKB Biotechnology) column (2 × 40 cm) equilibrated with chloroform-methanol-water, 30:60:10 (v/v). The neutral lipid fraction from the eluate was evaporated, redissolved, and then fractionated on a Florisil column (1 × 20 cm, 60–100 mesh; Floriden Co., New York, NY) by the method of Saito and Hakomori (27) with slight modification. The acetylated neutral glycolipid fraction and the acetylated SM fraction were deacetylated with 0.5 M KOH in methanol at 37 °C for 6 h. The yield of the deacetylated neutral glycolipid fraction containing ceramide mono-, di-, and trisaccharides was 2.7 mg. The deacetylated SM fraction was...
isolant, NCAM was purified on an AF11-Sepharose 4B column (5 ml). The suspension was centrifuged at 105,000 × g for 1 h, and the supernatant 2-week rat brains were homogenized with a Positron homogenizer containing 0.5% Nonidet P-40 to extract NCAM. After stirring for 1 h, the homogenate was centrifuged at 105,000 × g for 1 h, and the eluate fraction with a purification of about 2-fold.

The third step of the purification involved ASOR-conjugated Sepharose affinity chromatography. Among several buffers tested, a buffer containing 10 mM N-acetyllactosamine was effective for elution of the activity from the column. Because N-acetyllactosamine and glycerol inhibited the glucuronyltransferase activity, the apparent yield of the enzymatic activity in the eluate fraction was very low (9% that of the Nonidet P-40 extract; see Table I). However, after dialysis, the activity recovered to more than 30% that of the Nonidet P-40 extract. The purification achieved with this ASOR-Sepharose 4B affinity chromatography was over 1,000-fold.

The fourth step of the purification involved dye ligand affinity chromatography. A Matrex gel Blue A column was found to retain the glucuronyltransferase activity. 1 mM NaCl was effective for dissociating the enzyme from the column, with a 10-fold increase in the specific activity.

The fifth step of the purification involved Mono Q anion exchange chromatography. The glucuronyltransferase activity was mainly eluted in fractions 43–47 (Fig. 1), in which the concentration of NaCl was around 0.4 M. Because of the low amount of proteins in each fraction, protein quantification and SDS-PAGE were carried out with several (five to seven) fractions combined. Five-fold purification was achieved through this step. In the following purification steps (HiTrap chelating and HiTrap heparin columns), the protein concentrations were determined in the same way.

The sixth step of the purification involved HiTrap chelating metal chelate affinity chromatography. Mono Q eluate fractions 43–47 were pooled and applied to a HiTrap chelating column, which had been chelated with Cu²⁺. The glucuronyltransferase activity was eluted at a glycine concentration of 15 mM (fractions 22–28 in Fig. 2). Two-fold purification was achieved at this step.

The last step of the purification involved HiTrap heparin affinity chromatography, utilizing the inhibitory activity of heparin, as shown in Table II. The glucuronyltransferase bound to the column and was eluted in fractions 43–47 at a NaCl concentration of around 0.7 M (Fig. 3A). Upon SDS-PAGE, a major band of 45 kDa was observed, with a few minor bands. Since these minor bands were predominant components in the previous fractions, i.e., fractions 38–42, the HiTrap heparin affinity chromatography was repeated. The glucuronyltransferase thus obtained gave a single band corresponding to 45 kDa upon SDS-PAGE (Fig. 3B). The 45-kDa protein was shown to have a SH-group specifically protected by UDP-GlcA when the eluate fraction on Ma-
trex gel Blue A column chromatography was treated with N-maleimidopropionyl-biocytin (MPB) according to the procedure of Pukazhenthi et al. (31). Based on these results, the 45-kDa protein was tentatively concluded to be a glucuronyltransferase. This conclusion was finally confirmed by our recent successful cDNA cloning of a HNK-1-associated glucuronyltransferase on the basis of the partial amino acid sequence of the purified protein (32).

Thus, the glucuronyltransferase involved in the biosynthesis of the HNK-1 epitope on glycoprotein acceptors (GlcAT-P) was purified to apparent homogeneity from postnatal 2-week rat forebrains, with 1,200,000-fold purification and a 4.0% overall recovery (Table I).

FIG. 1. Mono Q column chromatography of the glucuronyltransferase fraction from Matrex gel Blue A chromatography. The eluate fraction from the Matrex gel Blue A column was dialyzed and then applied to a Mono Q column as described under “Experimental Procedures.” The eluate was collected as 0.5-ml fractions, and then the glucuronyltransferase activity was measured. Closed circles indicate the glucuronyltransferase activity, the dashed line the deduced concentration of NaCl, and open boxes the protein concentration. The fractions indicated by the bar were combined.

FIG. 2. HiTrap chelating metal chelate column chromatography of the glucuronyltransferase fraction on Matrex gel Blue A column chromatography. The combined eluate fraction obtained on Mono Q column chromatography was loaded onto a HiTrap chelating column, which had been pretreated with CuCl₂, and was then eluted as described under “Experimental Procedures.” The eluate was collected as 1-ml fractions, and then the glucuronyltransferase activity was measured. The fractions indicated by the bar were combined.

FIG. 3. HiTrap heparin column chromatography of the glucuronyltransferase fraction on HiTrap chelating metal chromatography. A, the eluate obtained on HiTrap chelating metal chromatography was loaded onto a HiTrap heparin column. The eluate was collected as 0.5-ml fractions. After dialysis, the eluate was applied to the second HiTrap heparin column as described under “Experimental Procedures.” The eluate was collected as 0.5-ml fractions, and then the glucuronyltransferase activity was measured. B, SDS-PAGE of the glucuronyltransferase fraction (fractions 38–42) under reducing conditions. The figures on the left indicate the sizes and positions of marker proteins.

TABLE II
Substrate specificity of the purified enzyme, as measured by inhibition assay

| Inhibitor            | Concentration | Inhibition |
|----------------------|---------------|------------|
| N-Acetyllactosamine  | 5 mM          | 95%        |
| Lacto-N-biose        | 5 mM          | 0.0%       |
| Lactose              | 5 mM          | 23%        |
| Nle-Cer              | 5 mM          | 13%        |
| Heparin              | 1.0 mg/ml     | 90%        |
| Heparan sulfate      | 1.0 mg/ml     | 36%        |
| Hyaluronic acid      | 1.0 mg/ml     | 9.5%       |
| Chondroitin          | 1.0 mg/ml     | 0.0%       |
| GlcA                 | 10 mM         | 0.0%       |
| UDP                  | 10 mM         | 98%        |
| UDP-GlcA             | 10 mM         | 99%        |
| UDP-GlcNAc           | 10 mM         | 67%        |
| CMP-NeuAc            | 10 mM         | 1.5%       |

Superose 12 gel filtration chromatography of the purified glucuronyltransferase indicated that the molecular mass of the enzyme was approximately 90 kDa, as shown in Fig. 4. This value is almost 2 times larger than that determined by SDSPAGE under reducing conditions (45 kDa, Fig. 3B), suggesting that the enzyme occurs as a homodimer of 45-kDa polypeptides under non-denaturing conditions.

Enzymatic Properties of GalAT-P: Substrate Specificity of GalAT-P—In order to study the substrate specificity of purified enzyme, we analyzed the effects of various compounds on the glucuronyltransferase activity toward ASOR (Table II). N-Acetyllactosamine at the concentration of 5 mM had a very potent (95%) inhibitory effect. In contrast, lacto-N-biose (Galβ1–3GlcNAc) and lactose (Galβ1–4Glc) had no or little effect on the enzymatic activity (0% and 23%, respectively), indicating that the enzyme recognizes not only the terminal sugars on the acceptor molecules but also the penultimate sugars and their linkage positions. Hyaluronic acid and chondroitin had little or no inhibitory effect (9.5% and 0%, respectively), whereas heparin and heparan sulfate decreased the
enzymatic activity (90% and 36%, respectively). These results may indicate that heparin and heparan sulfate act as acceptors for the enzyme but that hyaluronic acid and chondroitin do not. However, the purified enzyme did not show any transferase activity toward any of these glycosaminoglycans (data not shown).

With regard to donor specificity, UDP and UDP-GlcA exhibited strong inhibitory effects (99% and 98%, respectively), followed by UDP-GlcNAc (67%). In contrast, GlcA and CMP-NeuAc had no or little effect on the activity (0.0% and 1.5%, respectively). These results suggest that the enzyme principally recognizes the terminal non-reducing N-acetyllactosamine structure in the sugar chains on glycoproteins and the nucleotide portion of UDP-GlcA.

In order to determine the effect of the polypeptide portion of the acceptor glycoconjugates, various asialo-glycoproteins and glycolipids were tested as acceptors, as described under "Experimental Procedures." The purified enzyme sufficiently transferred the glucuronic acid to asialo-NCAM and asialofetuin (72% and 87% of that in the case of ASOR, respectively). In contrast, asialo-thyroglobulin, which contains high manose-type sugar chains, as well as complex-type sugar chains, was a poor acceptor of the enzyme. Interestingly, GlcAT-P did not show any activity toward paragloboside, a precursor glycolipid of the HNK-1 epitope.

**Kinetic Analysis and Divalent Cation Dependence of GlcAT-P**—The dependence of the rate of the glucuronyltransferase reaction on the concentrations of ASOR and UDP-GlcA was examined, and their kinetic parameters were analyzed by Lineweaver-Burk plotting (data not shown). The $K_m$ values for ASOR and UDP-GlcA were 1.9 and 22 $\mu$m, respectively. The $V_{max}$ value of the enzyme (4.5 units/mg) is comparable to those reported for the purified glycosyltransferases from the Golgi apparatus, such as $G_{\text{M2}}/G_{\text{D2}}$ N-acetylgalactosaminyltransferase (3.6 units/mg) (33), $\alpha_2,6$-sialyltransferase (8.2 units/mg) (34), $\alpha_1,3$-galactosyltransferase (4.3 units/mg) (35), and $\beta_1,2$-N-acetylgalactosaminyltransferase (28 units/mg) (36).

The effects of various divalent cations on the glucuronyltransferase activity were measured as described under "Experimental Procedures" in the presence of various amounts of lipids purified from the Folch lower phase. The data indicate the amounts of lipids required to give 50% of the full activity observed in the presence of a saturating amount of the heat-treated Nonidet P-40 extract.

**TABLE III**

| Lipid                  | Amount of lipid giving 50% activation |
|------------------------|--------------------------------------|
| Sphingomyelin (16:0)   | 5.2                                   |
| Phosphatidylcholine     | 91.4                                  |
| Phosphatidylethanolamine| >600                                  |
| Phosphatidylserine      | >1200                                 |
| Phosphatidilinositol    | >1200                                 |
| Neutral glycolipids     | >1200                                 |

**TABLE IV**

| Lipid                  | Amount of lipid giving 50% activation |
|------------------------|--------------------------------------|
| Sphingomyelin (16:0)   | 4.2                                   |
| Sphingomyelin (18:0)   | 3.8                                   |
| Sphingomyelin (18:1)   | >100                                  |
| Sphingomyelin (24:0)   | 10.5                                  |
| Sphingomyelin (24:1)   | >100                                  |
| Ceramide               | >100                                  |

**Fig. 4.** The elution profile of the purified glucuronyltransferase on a Superose 12 gel filtration column. The purified glucuronyltransferase was applied to a Superose 12 gel filtration column, and then the glucuronyltransferase activity of each eluate fraction was measured. The arrowhead indicates the void volume. Arrows indicate the positions of aldolase (Ald, 158 kDa), bovine serum albumin (BSA, 67 kDa), ovalbumin (Ova, 43 kDa), chymotrypsinogen A (Chy, 25 kDa), and ribonuclease (Rib, 13.7 kDa). The glucuronyltransferase activity was eluted at the position of a 90-kDa protein. The inset shows estimation of the molecular weight of the glucuronyltransferase.

**Fig. 5.** Effects of various rat brain extracts on the partially purified glucuronyltransferase. A, the glucuronyltransferase activity of the eluate fraction obtained on Matrex gel Blue A column chromatography was measured in the presence or absence of various rat brain extracts. Brain extracts: NE, heat-inactivated 2% Nonidet P-40 extract; FU, Folch upper phase of postnatal 14-day rat forebrain; FL, Folch lower phase of postnatal 14-day rat forebrain. B, the effect of the Folch lower phase on the glucuronyltransferase activity. The activity was compared with the control value in the presence of the heat-treated Nonidet P-40 extract.
transferase activity were determined. Among those tested, Mn²⁺ activated the enzyme most effectively. Co²⁺ and Mg²⁺ showed 20% and 14% of the activity of Mn²⁺, respectively. Ca²⁺, Ba²⁺, Ni²⁺, Cu²⁺, and Zn²⁺ had no effect on the enzyme at all (data not shown).

GlcAT-P Required SM as an Activator—To our surprise, during the process of purification, the enzyme activity disappeared almost completely at the step of Matrex gel Blue A affinity chromatography (with approximately 22,500-fold purification). However, the activity was recovered when an aliquot of a Nonidet P-40 extract was added to the assay mixture, suggesting that GlcAT-P requires some kinds of activator(s) for its catalytic activity. This presumed activator was stable on heating at 100 °C for 3 min. Therefore, we carried out the enzyme assay in the presence of a saturating amount of the heat-treated Nonidet P-40 extract in the following purification steps, as described under “Experimental Procedures.”

We tried to identify the activator present in postnatal 14-day rat brains. First, we found that the chloroform-methanol extract of rat brains can substitute for the heat-inactivated Nonidet P-40 extract. Upon Folch partitioning (26), the organic solvent layer (Folch lower phase) activated the enzyme effectively in a saturable manner, but the upper phase did not (Fig. 5, A and B). These lines of evidence indicated that the activator is a kind of lipid. Then, the respective lipid components were prepared from the Folch’s lower phase according to the procedure described under “Experimental Procedures,” and their stimulatory activity was measured. Table III shows the amount of each lipid that gives 50% of the full activity (in the presence of heat-treated Nonidet P-40 extract). It is clear that SM caused recovery of the enzymatic activity most effectively (5.2 μg for 50% recovery), followed by phosphatidylcholine, which has phospho-}

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