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Characterization of a glucuronyltransferase: neolactotetraosylceramide glucuronyltransferase from rat brain

CHIKA KAWASHIMA, KOJI TERAYAMA, MASAYUKI II, SHOGO OKA and TOSHISUKE KAWASAKI*

Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-01, Japan

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The properties of a rat brain glucuronyltransferase, which is presumed to be associated with the biosynthesis of the HNK-1 epitope on sulfoglucuronyl glycolipids, are described. The enzyme required divalent cations for reaction, with maximal activity at 10 mM Mn^{2+}, and exhibited a dual optimum at pH 4–5 and pH 6 depending upon the buffer used, with the highest activity at pH 4.5 in MES buffer. This enzyme strictly recognized the Galβ1-4GlcNAc terminal structure, and was highly specific for neolacto (type 2) glycolipids as acceptor. The enzyme was localized specifically in the brain, and was barely detected in other issues, including the thymus, spleen, liver, kidney, lung, and sciatic nerve fibres. Phosphatidylinositol and phosphatidylserine increased the enzymatic reaction 4.4- and 2.3-fold, respectively, whereas phosphatidylcholine slightly decreased the rate.

Keywords: glucuronyltransferase, HNK-1 antigen, rat brain

Abbreviations: GlcA, glucuronic acid; Lc-PA_{14}, lactotetraose-phenyl-$C_{14}H_{29}$; nLc-PA_{14}, neolactotetraose-phenyl-$C_{14}H_{29}$; nLcOse$_4$-Cer, neolactotetraosylceramide; NP-40, Nonidet P-40; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SGGL, sulfoglucuronyl glycolipid.

Carbohydrate moieties on cell surface molecules have been thought to play important roles in cell-to-cell interaction events [1–3]. With the progress in immunological methods such as generation of monoclonal antibodies, neural specific carbohydrate epitopes have been discovered [4]. Among them, the HNK-1 carbohydrate epitope, which is recognized by the monoclonal antibody HNK-1 [5], is found in many neural adhesion molecules of the immunoglobulin superfamily [6], such as neural cell adhesion molecules (N-CAM) [7], L1 [7], and J1 [8], myelin-associated glycoprotein (MAG) [9, 10], transiently-expressed axonal glycoprotein-1 (TAG-1) [11], and Po [12]. This carbohydrate epitope is expressed not only on glycoproteins but also on sulfoglucuronyl glycolipids (SGGLs) [13–15]. The SGGLs are temporally and spatially regulated molecules present in the nervous system during its development [16,17]. The structures of these glycolipids have been determined as 3-sulfoglucuronyl neolactotetraosyl ceramide (SGGL-1), HSO_3-3GlcAβ1-3Galβ1-4GlcNAcβ1-3Galα1-4Glcβ1-1Cer, and 3-sulfoglucuronyl neolactohexaosyl ceramide (SGGL-2), HSO_3 - 3GlcAβ1 - 3Galβ1 - 4GlcNAcβ1 - 3Galβ1 - 4Glc - NAcβ1-3Galβ1-4Glcβ1-1Cer, respectively [18–20]. The terminal sulfate-3-glucuronyl moiety is essential for immunoreactivity with the HNK-1 antibody [14]. Not only the HNK-1 antibody [21, 22] but also HNK-1 reactive glycolipids (SGGLs) interfere with neural cell interactions [23], which suggests that the HNK-1 carbohydrate epitope plays a crucial role in cell-to-cell adhesion and recognition. It should be noted that only a subpopulation of the many molecules known to carry the HNK-1 epitope expresses the HNK-1 epitope, and the rest do not [7]. This is the case for N-CAM [7], MAG [7, 10], L1 [24], and Po [25] and PI-GP150 [26]. PI-GP150 is a novel phosphatidyl inositol-anchored glycoprotein, whose HNK-1 epitope varies from region to region in the rat brain, even though the PI-GP150 protein is expressed almost evenly throughout the brain [26]. These findings suggest that the HNK-1 epitope plays an important role independently of the polypeptide portion of the molecule in the cell adhesion function of the protein.

To investigate the expression and regulation mechanism of the HNK-1 epitope in the nervous system, we decided to study glycosyltransferase(s) involved in the biosynthesis of this epitope. We have established an assay system and detected the glucuronyltransferase activity specifically expressed in the brain. The enzyme was different from previously reported hepatic glucuronyltransferase, and was

* To whom correspondence should be addressed.

presumed to be involved in the biosynthesis of the HNK-1 epitopes on SGGLs. Similar glucuronyltransferases in embryonic chicken brain and rat brain have been described [27, 28]. We confirm these observations and reveal some unique properties of the glucuronyltransferase.

Materials and methods

Materials

UDP-[^{14}C]glucuronic acid was purchased from ICN Radiochemicals, CA, USA. Synthetic glycolipid nLc-PA_{14} (neolactotetraose-PA_{14}; nLcOse$_4$-phenyl-$C_{14}H_{29}$), Lc-PA_{14} (lactotetraose-PA_{14}; LcOse$_4$-phenyl-$C_{14}H_{29}$), and the crude ganglioside mixture were obtained from BioCarb Chemicals, Lund, Sweden; nLcOse$_4$Cer (neolactotetraosylceramide) was from Dia-Iatron (Tokyo, Japan); L-α-phosphatidylinositol (soybean), L-α-phosphatidylethanolamine (soybean), L-α-phosphatidylcholine (egg yolk), and L-α-phosphatidyl-L-serine were from Wako Chemicals (Osaka, Japan); β-glucuronidase (limpets) and LacCer (lactosyl ceramide) were from Sigma Chemical Co (St. Louis, USA). High performance TLC plates (HPTLC, silica gel 60) were from E. Merck (Darmstadt, Germany).

Preparation of the enzyme

Wistar rats of various ages were killed and the brains were homogenized with five volumes of 0.32 M sucrose in 10 mM HEPES buffer, pH 6.5, containing 1 mM EDTA, 0.1% 2-mercaptoethanol, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} pepstatin, 100 μg ml^{-1} benzamidine, and 1 mM PMSF (phenylmethanesulfonyl fluoride) (buffer A). After the homogenate was centrifuged at 105 000 × **g** for 1 h, the resulting pellet was suspended in five volumes of 2% NP-40 in buffer A. The suspension was stirred with a magnetic stirrer for 1 h to extract the enzyme, and was then recentrifuged at 105 000 × **g** for 30 min. The supernatant was used as the enzyme sample. All these steps were performed at 4 °C. For stabilization, in some cases, glycerol was added to the supernatant to give a final concentration of 20%. Under these conditions, the enzyme could be stored at −20 °C for at least two months without loss of activity.

Glucuronyltransferase assay

Glucuronyltransferase was assayed using a synthetic glycolipid, nLc-PA_{14} as an acceptor. Unless otherwise noted, the standard assay mixture contained the following components in a total volume of 50 μl; nLc-PA_{14} (7.5 nmol), UDP-[^{14}C]glucuronic acid (5–20 nmol, 0.65–2.6 × 10^7 counts min^{-1} μmol^{-1}), enzyme protein (100–200 μg), MES buffer, pH 4.5 (10 μmol), $MnCl_2$ (0.5 μmol), ATP (0.5 μmol), and NP-40 (100–200 μg). The reaction mixtures were incubated for 3 h at 37 °C, and the reactions were terminated by addition of 20 volumes of chloroform–methanol (2:1 by vol). The radioactive reaction products were separated from the labeled precursor according to the procedure of Fishman *et al.* [29]. The reaction mixture was applied to a column (0.5 cm × 5.0 cm) of Sephadex G-25 which had been equilibrated with chloroform–methanol–water (60:30:4.5 by vol). The column was then washed with 5 ml of the same solvent, and the effluent and washings were collected in a scintillation vial and evaporated to dryness by gentle warming. The radioactivity of the sample was counted using a liquid scintillation counter (Beckman LS-6000). The enzyme activity was expressed as the amount of incorporated radioactive glucuronic acid into the exogenous acceptor per mg protein per hour of incubation. The protein concentration was determined by the method of Bradford [30].

β-Glucuronidase digestion

The radioactive product obtained as above was dissolved in 200 μl 10 mM sodium acetate buffer, pH 3.8, containing 400 units of β-glucuronidase from limpets (*Patella vulgata*) and the mixture was incubated overnight at 37 °C. After centrifugation of the reaction mixture, the supernatant was spotted on an HPTLC plate (1.5 cm × 9.0 cm) and the plate was developed in n-propanol–28% aq.NH_3–water (75:5:25 by vol). The radioactivity of silica gel which was removed from the plate at intervals of 5 mm was measured in the liquid scintillation counter.

Results

Characterization of reaction product

Upon HPTLC analysis, the effluent fraction from Sephadex G-25 chromatography showed a single spot migrated slightly slower than nLc-PA_{14}, as shown in Fig. 1.

When the radioactive reaction product was digested with β-glucuronidase from limpets, the radioactive spot observed before the digestion disappeared and a new spot appeared at the position corresponding to that of glucuronic acid (data not shown). These results indicated that glucuronic acid was in fact transferred to nLc-PA_{14} through a β-linkage.

Effect of pH on glucuronyltransferase activity

As Fig. 2 shows, the optimal pH was between 4.0 and 4.5. No enzyme activity was detected at any pH when the exogenous acceptor was not added to the reaction mixture. The enzyme activity was affected markedly by the kind of buffer used. MES buffer gave about twofold higher activity than sodium acetate buffer at pH 4.5 (Fig. 2). In sodium phosphate buffer (pH 4.5) or sodium citrate buffer (pH 4.5), the activity was hardly detected. At the end of this study, Chou *et al.* reported that the pH optimum of a similar enzyme in rat brain was 6.0 in Na-cacodylate buffer [28]. This was confirmed in the present study, and the results are

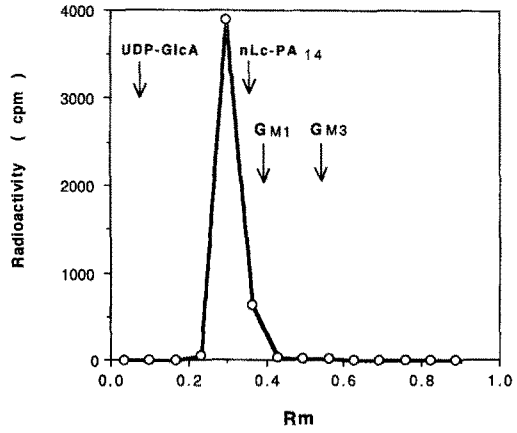

Figure 1. Identification of [^{14}C]GlcA-nLc PA_{14} on HPTLC. About 5000 disintegrations min^{-1} (125 pmol) ^{14}C-labeled reaction product recovered from Sephadex G-25 column was spotted along with authentic standards (UDP-GlcA, $nLcPA_{14}$, GM1, and GM3). The plate was developed in a solvent system of n-propanol–28% NH_4OH–H_2O (75: 5: 25 by vol). The lane spotted with the reaction product was scraped at 5 mm intervals and the radioactivity was measured using a toluene scintillation system. Standards were visualized by spraying orcinol sulfuric acid reagent [36]. The migrated positions of the reaction product and standards were indicated relative to the solvent front (R_m).

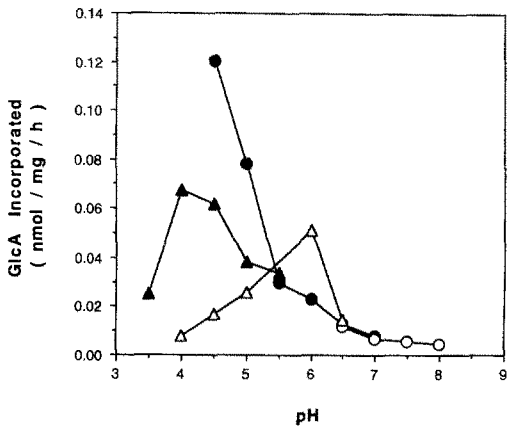

Figure 2. Effect of pH on the activity of rat brain glucuronyltransferase. The reaction mixture contained the following components in a final volume of 50 µl; nLc-PA_{14}, 7.5 nmol; UDP-[^{14}C]GlcA, 5 nmol (2.6×10^7 counts min^{-1} μmol^{-1}); NP-40 extract of adult rat brain, 200 µg; buffers (▲, sodium acetate 5 µmol, in the pH range 3.5–5.5, ●, MES-NaOH, 10 µmol, pH 4.5–7.0, ○, HEPES-NaOH, 10 mmol, pH 6.5–8.0, △, sodium cacodylate buffer, pH 4.0–6.5); $MnCl_2$, 0.5 µmol; ATP, 0.5 µmol. The mixture was incubated for 3 h at 37 °C. The amount of [^{14}C]GlcA transferred to the acceptor was assayed as described in the Materials and methods section.

also shown in Fig. 2. However, the specific activity of the enzyme was much higher at pH 4–4.5 in MES buffer than that at pH 6.0 in Na-cacodylate buffer, and we used MES buffer, pH 4.5, in the following enzyme assays.

Effect of divalent cations

Divalent cations were essential for the enzymatic reaction, and the presence of EDTA at 10 mM abolished the activity completely. As shown in Fig. 3(a), Mn^{2+} gave the highest activity under the standard conditions. Zn^{2+} and Mg^{2+} were 64% and 16% as effective as Mn^{2+}, respectively. The optimal concentration of Mn^{2+} (at pH 4.5) was about 10 mM (Fig. 3(b)).

Kinetic studies

Under the standard assay conditions (pH 4.5), product formation was proportional to protein concentration and time of incubation up to 1.5 mg and 3 h, respectively (Fig. 4). The K_m and V_{max} values for UDP-glucuronic acid were 0.20 mM and 0.23 nmol per mg protein per h, respectively (Fig. 5). On the other hand, these values at pH

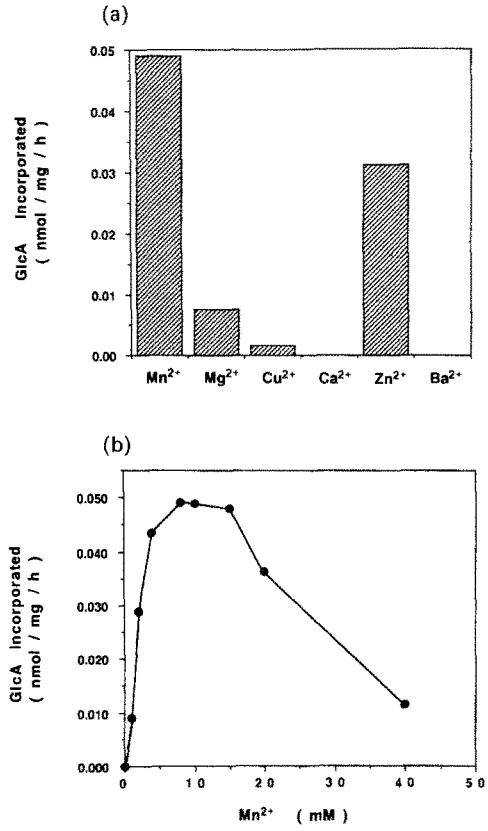

Figure 3. Dependence of rat brain glucuronyltransferase on divalent cations. (a) The reaction mixture contained the following components in a final volume of 50 µl; nLc-PA_{14}, 7.5 nmol; UDP-[^{14}C]GlcA, 5 nmol (2.6×10^7 counts min^{-1} μmol^{-1}); NP-40 extract of rat brain, 150 µg; MES buffer, pH 4.5, 10 µmol; divalent cations (Mn^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, Ca^{2+}, Ba^{2+}), 0.5 µmol; ATP, 0.5 µmol. The mixture was incubated for 3 h at 37 °C. The amount of [^{14}C]GlcA transferred to the acceptor was assayed as described in the Materials and methods section. (b) The reaction mixture contained various amounts of Mn^{2+} as divalent cation. Other conditions of the reaction were the same as in (a).

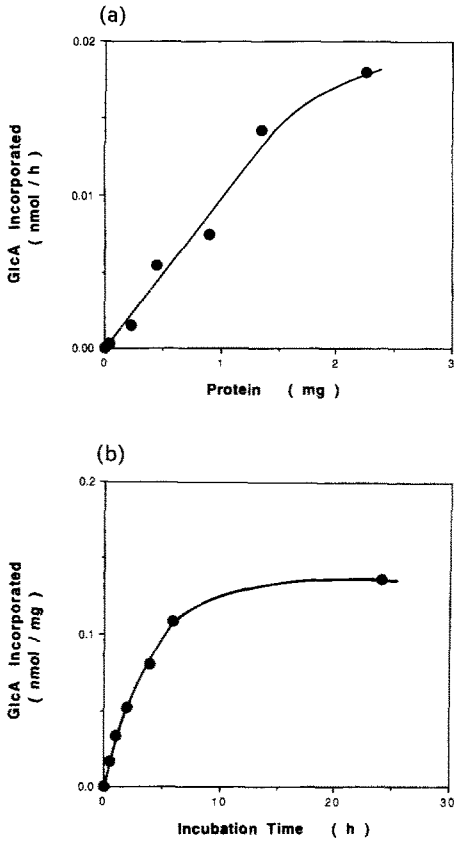

Figure 4. Dependence of the rat brain glucuronyltransferase on the amount of homogenate protein and the time of incubation. (a) The reaction mixture contained the following components in a final volume of 50 µl; nLc-PA_{14}, 7.5 nmol; UDP-[^{14}C]GlcA, 0.17 nmol (3.6×10^8 counts min^{-1} μmol^{-1}); adult rat homogenate protein, 0–2.25 mg; MES buffer, pH 4.5, 10 µmol; $MnCl_2$, 0.5 µmol; ATP, 0.5 µmol. The mixture was incubated 3 h at 37 °C. The amount of [^{14}C]GlcA transferred to the acceptor was assayed as described in the Materials and methods section. (b) The reaction mixture contained UDP-[^{14}C]GlcA, 5 nmol (2.6×10^7 counts min^{-1} μmol^{-1}); NP-40 extract of rat brain, 130 µg, and the other components were the same as in (a). The mixtures were incubated for various times and the amount of [^{14}C]GlcA transferred was assayed as described in (a).

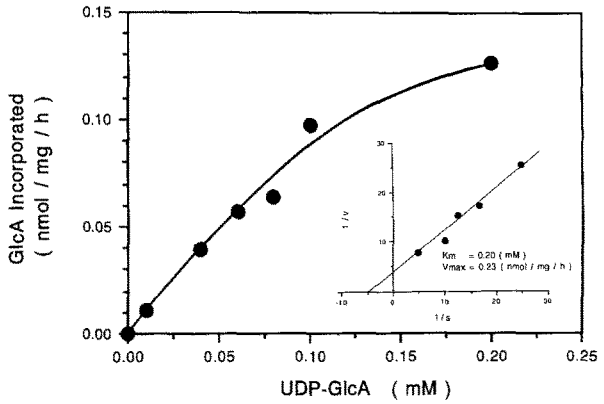

Figure 5. Dependence of rat brain glucuronyltransferase on the concentration of UDP-glucuronic acid. NP-40 extract of adult rat brain (150 µg) was used as the enzyme source. Different concentrations of UDP-[^{14}C]GlcA were added to the standard assay mixture, and the assay was carried out as described in the Materials and methods section. The inset shows the data analyzed with a Lineweaver-Burk plot.

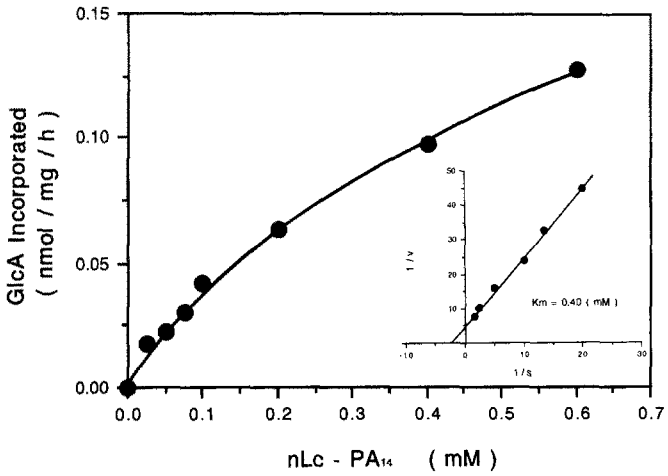

Figure 6. Dependence of rat brain glucuronyltransferase on the concentration of nLc-PA_{14}. NP-40 extract of adult rat brain (150 µg), UDP-[^{14}C]GlcA (5 nmol, 2.6×10^7 counts min^{-1} μmol^{-1}), and different concentrations of nLc-PA_{14} were added to the standard assay mixture. The assay was carried out as described in the Materials and methods section.

6.5 in HEPES buffer were 0.057 mM and 0.033 nmol per mg protein per h, respectively (data not shown). The K_m values for nLc-PA_{14} (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA_{14}) was determined to be 0.40 mM at pH 4.5 (Fig. 6), which is higher than the value at pH 6.5 (0.22 mM). These findings suggest that the affinity between the enzyme and the substrates (sugar nucleotide and glycolipid acceptor) is higher at pH 6.5, though the turnover occurs more rapidly at pH 4.5 under the assay conditions used.

Acceptor specificity

As shown in Table 1(a), $nLcOse_4Cer$ (paragloboside; Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer), the putative endogenous acceptor substrate of this enzyme, as well as nLc-PA_{14}, was a good acceptor for glucuronic acid. On the other hand, transfer of glucuronic acid to a synthetic glycolipid Lc-PA_{14} (Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA_{14}) was barely detected. In addition, a ganglioside mixture was also inactive. Competition experiments, shown in Table 1(b), indicated that *N*-acetyllactosamine (Galβ1-4GlcNAc) and neolactotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) did not inhibit the transfer of GlcA to the glycolipid acceptor, nLc-PA_{14}, which suggests that the enzyme requires the presence of a hydrophobic portion in the acceptor molecules.

Table 1. Acceptor specificity of rat brain glucuronyltransferase. NP-40 extract of adult rat brain (80 μg), UDP-[^{14}C]GlcA (20 nmol, 0.65 × 10^7 counts min^{-1} μmol^{-1}) and various glycolipids were added to the standard assay mixture as an acceptor and the assay was carried out as described in the Materials and methods section. (a) nLcOse$_4$Cer (7.5 nmol, 11.8 μg; Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer), nLc-PA$_{14}$ (7.5 nmol), Lc-PA$_{14}$ (7.5 nmol; Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA$_{14}$), LacCer (7.5 nmol; Galβ1-4Glcβ1-1Cer), or crude ganglioside mixture (1 mg; containing 0.4% GM3, 1.0% GD3, 1.5% GM2, 13.0% GM1, 38.0% GDIa, 9.0% GDIb, 16.0% GTIb, 1.0% GQIb, and 20% other gangliosides) was added to the standard reaction mixture as an acceptor for the glucuronyltransferase. (b) *N*-Acetyllactosamine (75 nmol; Galβ1-4GlcNAc) or nLcOse$_4$ (75 nmol; Galβ1-4GlcNAcβ1-3Galβ1-4Glc) was added with or without nLc-PA$_{14}$ (7.5 nmol) to the standard assay mixture and the assay was carried out as in (a).

(a)	*Glycolipid*	*Activity (%)*
	nLcOse$_4$-Cer	100
	nLcPA$_{14}$	103
	Lc-PA$_{14}$	11
	Lac-Cer	0
	Gangliosides	0

(b)	*Acceptor (mM)*			*Activity (%)*
	nLc-PA$_{14}$	*Galβ1-4GlcNAc*	*nLc*	
	0.15	–	–	100
	0.15	1.5	–	107
	0.15	–	1.5	108
	–	–	1.5	0

Tissue distribution and regional distribution in brain

Figure 7(a) shows the specific activity of the UDP-glucuronyltransferase in different rat tissues. The most predominant activity was found in the brain, including cerebral cortex and cerebellum, and little activity was detected in the thymus, spleen, sciatic nerve fiber or lung under the assay conditions. The homogenate of the liver and kidney showed significant activity. However, the enzyme in the liver and kidney transfers glucuronic acid not only to nLc-PA$_{14}$ but also to Lc-PA$_{14}$ with almost equal activity (data not shown). Therefore, the glucuronyltransferase activity detected in the liver and kidney was clearly distinguishable from the enzyme in the brain tissue.

Furthermore, we investigated the regional distribution of the enzyme in adult rat brain (Fig. 7(b)). The cerebral cortex and hippocampus expressed the glucuronyltransferase activity most strongly. The rest of the regions tested, viz., olfactory bulb, striatum, thalamus, cerebellum, midbrain, pons and medulla oblongata, also expressed the activity, but the activity levels of these regions were lower than that of the cerebral cortex.

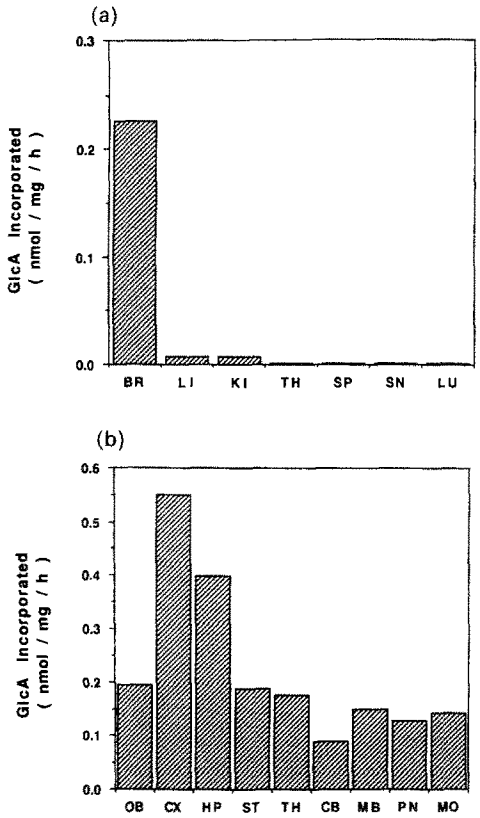

Figure 7. Tissue distribution and regional distribution in rat brain of the glucuronyltransferase. The homogenates of various tissues from adult rats were used as enzyme sources (100–360 μg protein). UDP-[^{14}C]GlcA (5 nmol, 2.6 × 10^7 counts min^{-1} μmol^{-1}) was added to the standard assay mixture as described in the Materials and methods section. (a) Tissue distribution; BR, brain; LI, liver; KI, kidney; TH, thymus; SP, spleen; SN, sciatic nerve fiber; LU, lung. (b) Regional distribution in rat brain; OB, olfactory bulb; CX, cerebral cortex; HP, hippocampus; ST, striatum; TH, thalamus; CB, cerebellum; MB, midbrain; PN, pons; MO, medulla oblongata.

Effect of development stages

The glucuronyltransferase activity of rat cerebral cortex and cerebellum in several developmental stages was investigated. As shown in Fig. 8, the cerebral cortex showed the maximal activity at postnatal day 9 (P9). In the cerebellum, the glucuronyltransferase activity was at the highest level at embryonic day 20 (E20) within the developmental stages examined here. The activity then declined but never disappeared completely and remained at certain levels for a considerable period of time in the cerebral cortex and cerebellum, respectively.

Effect of phospholipids

The effect of phospholipids were examined by the addition of phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) to the standard incubation mixture. The UDP-glucuronyltransferase activity was affected variously by these

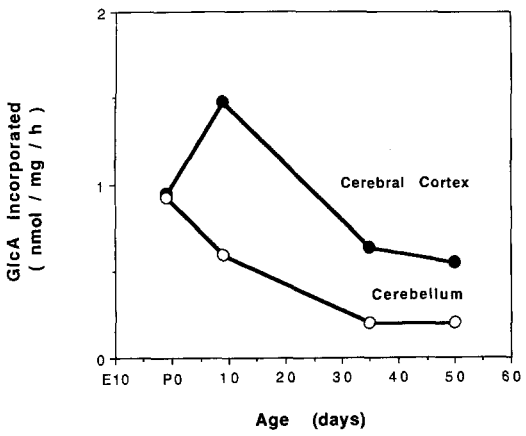

Figure 8. Developmental changes of the rat brain glucuronyltransferase activity. Homogenates of the rat brain (160–200 μg protein) in various stages of development (E20, P9, P35, P50), UDP-[^{14}C]GlcA (5 nmol, 2.6 × 10^7 counts min^{-1} μmol^{-1}) and phosphatidylinositol (50 μg) were added to the standard assay mixture, and assay was carried out as described in the Materials and methods section. ●, Cerebral cortex; ○, cerebellum.

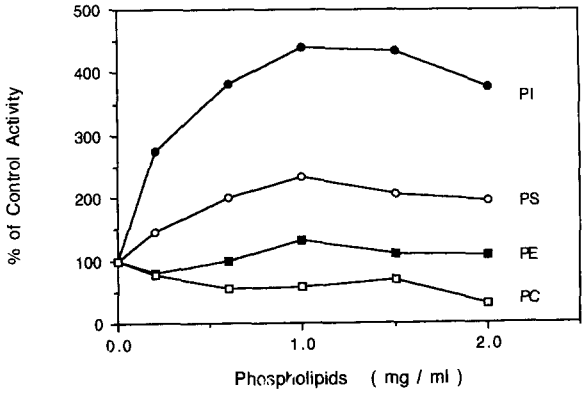

Figure 9. Effect of phospholipids on the activity of rat brain glucuronyltransferase. NP-40 extract of adult rat brain (80 μg), UDP-[^{14}C]GlcA (20 nmol, 0.65 × 10^7 counts min^{-1} μmol^{-1}) and various phospholipids were added to the standard assay mixture, and the assay was carried out as described in the Materials and methods section. ●, PI, Phosphatidylinositol; ○, PS, phosphatidylserine; ■, PE, phosphatidylethanolamine; □, PC, phosphatidylcholine.

phospholipids (Fig. 9). The addition of PI or PS promoted the transfer reaction and the highest activities were found at 1 mg per ml phospholipid. Phosphatidylinositol, which is the best activator tested, increased the transfer of glucuronic acid about 4.4-fold. Similarly, phosphatidylserine could activate the reaction about 2.3-fold. On the other hand, phosphatidylcholine inhibited the reaction slightly and phosphatidylethanolamine did not show any significant effect on the reaction.

Furthermore, the addition of the phospholipid fraction of the whole brain, prepared by the procedure of Folch *et al.* [31] to the standard assay mixture resulted in the increase of the activity of the NP-40 extract up to 10 times the control value added (data not shown).

Discussion

We have described the properties of a glucuronyltransferase in rat brain, which transfers glucuronic acid to nLcOse$_4$-Cer. Previously, several UDP-glucuronyltransferases have been reported. Liver glucuronyltransferases are associated with detoxification of many xenobiotic compounds [32]. An olfactory glucuronyltransferase, which is located in the olfactory epithelium, seems to be involved in odorant signal termination [33]. The glucuronyltransferase studied here is localized strictly in the brain, and was expressed widely in various parts of the brain (Fig. 6(a,b)). Therefore, this enzyme should be different from hepatic and olfactory glucuronyltransferases.

The enzyme was specific for neolacto (type 2) glycolipids and the lacto (type 1) glycolipid was inert as an acceptor (Table 1(a)). Thus, a synthetic glycolipid, Lc-PA$_{14}$ was only 10% as effective as nLc-PA$_{14}$. These two synthetic glycolipids have the same sugar compositions and sequences except for the linkage position of the terminal galactose residue, i.e., type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc). In addition, this enzyme did not transfer glucuronic acids to lactosylceramide (Galβ1-4Glc-Cer) or various gangliosides (Table 1(a)) despite the fact that nLcOse$_4$-Cer is an excellent acceptor. Therefore, the glucuronyltransferase reported here recognized the carbohydrate structure of Galβ1-4GlcNAc strictly, and specifically transferred the glucuronic acid to this carbohydrate moiety. This high specificity for neolacto glycolipids and localization of the activity in the brain suggests that this nLcOse$_4$-Cer: glucuronyltransferase is involved in the biosynthesis of the HNK-1 epitope.

Recently, similar glucuronyltransferases in embryonic chicken brain and rat brain have been described [27, 28]. However, these enzymes are slightly different in their requirement for cations and optimal pH. Although Mn^{2+} was essential for optimal activity for all these enzymes, Mn^{2+} could be replaced by Cd^{2+} (100%), Mg^{2+} (80%) and Co^{2+} (60%) for the chicken brain enzyme, and Mg^{2+} and Zn^{2+} were not effective at all for the rat brain enzyme described by Chou *et al.* [28]. By contrast, for the enzyme described here, Zn^{2+} and Mg^{2+} were 64% and 19% as effective as Mn^{2+}, while Cd^{2+} was not effective at all (Fig. 3). In addition, the effect of pH on this enzyme is unique. It appears to have a dual optimum, pH 4–4.5, and pH 6.0, depending upon the buffer used (Fig. 2). The kinetic parameters of the interaction between the enzyme and the substrate were measured at both pHs. The K_m values for UDP-GlcA and nLc-PA$_{14}$ were 0.20 mM and 0.40 mM at pH 4.5, but 0.057 mM and 0.22 mM at pH 6.5, respectively. On the other hand, the V_{max} value at pH 4.5 was several-fold larger than that at pH 6.5. The physiological significance of this unique pH dependency of the enzyme is not clear.

However, it could be an important regulatory factor for the enzyme at a particular local environment within the cells in which the enzyme is active.

The enzyme activity changes with the developmental stage of the rat brain. The activity was high during the pre- and perinatal periods, and then declined. Certain levels of activity were maintained through adulthood, but the level of the cerebral cortex was higher than that of the cerebellum. This corresponded well with the previous reports [28]. SGGL-1 and SGGL-2, HNK-1-reactive glycolipids, have been reported to have almost completely disappeared in the cerebral cortex by postnatal day 20 whereas, in the cerebellum, SGGLs showed increasing expression with postnatal development [16, 17, 28]. This may be explained by the presumption that the transfer of glucuronic acid is not the rate limiting step in the synthesis of SGGLs. The preceding step, formation of the glycolipid acceptor, or the final stage of SGGL synthesis, transfer of sulfate, could be the rate limiting step. In fact, Chou *et al.* suggested that the availability of the precursor glycolipid can be a regulatory factor for the synthesis of SGGL [28]. On the other hand, an alternative possibility that some other environmental factor, which controls the glucuronyltransferase activity, regulates the SGGLs expression should also be considered. In view of the fact that phospholipids have been known to affect the activity of the hepatic UDP-glucuronyltransferase [34, 35], we examined the effect of the phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC) on the glucuronyltransferase in the brain. PI and PS, which are negatively charged, increased the enzymatic activity 4.4- and 2.3-fold, respectively, at their optimal concentrations, whereas PC slightly reduced the activity (Fig. 8). It should be noted here that hepatic UDP-glucuronyltransferase is activated by positively charged lipids like phosphatidylcholine [34]. These findings suggest that some factors such as phospholipids control the expression of the HNK-1 epitope by regulating the activity of the glucuronyltransferase.

The glucuronyltransferase studied here seems to be involved in the biosynthesis of the HNK-1 epitope on SGGLs. It is not clear whether the enzyme also takes part in the synthesis of the HNK-1 carbohydrate on glycoproteins. The substrate specificity study showed that neolactotetraose (nLcOse$_4$: Galβ1-4GlcNAcβ1-3Galβ1-4Glc, 1.5 mM) and *N*-acetyllactosamine did not inhibit the transfer of glucuronic acid to nLc-PA$_{14}$, even at a 10-fold concentration (Table 1(b)). Thus, the glucuronyltransferase requires the hydrophobic moiety of the acceptor to be recognized. Our preliminary experiments indicated that asialoorosomucoid, which also has a Galβ1-4GlcNAc structure at its terminals, is active as an acceptor for glucuronyltransferase present in the NP-40 extract of the brain. However, the activity for asialoorosomucoid was separated from that for glycolipids using affinity chromatography [37]. These findings suggest that the glucuronyltransferase for glycolipids differs from that for glycoproteins. It will be interesting to study the possibility of differential regulation of the HNK-1 carbohydrate expression on glycoproteins and glycolipids and their roles in cell–cell interaction during the development of the nervous system.

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