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α1,3 Fucosyltransferase, α-L-fucosidase, α-D-galactosidase, β-D-galactosidase, and Le^x glycoconjugates in developing rat brain

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Fucosyltransferases (FTs) and various glycosidases that are involved in the biosynthesis or degradation of SSEA-1 (Le^x) antigens and their precursors in the CNS are developmentally regulated. In forebrain and cerebellum with lactosamine (LacNAc) as acceptor the FT activity was maximal at P15–P22, but with the glycolipid substrate paragloboside (nLc_4) the maximal activity in cerebellum was obtained at P10–P15. The FT activity, with these substrates, was insensitive to *N*-ethylmaleimide (NEM) and the glycolipid product had an α1,3 linkage (Fuc to GlcNAc) suggesting similarities of the investigated enzyme to the cloned human and rat FT IV. However, the observation of different patterns of FT activity in isoelectrofocused fractions (pH 3.5–10) with different types of acceptors, and the differential expression of Le^x containing glycolipids and glycoproteins during development strongly suggest the presence of more than one type of FT during development. Data on developmental expression of the hydrolytic enzymes, α-L-fucosidase, β-D-galactosidase and α-D-galactosidase, which can potentially hydrolyse SSEA-1 or its precursors, support the notion that SSEA-1 expression is the result of a dynamic balance between the activity of transferases and hydrolases.

Keywords: **α1,3 fucosyltransferase, SSEA-1, Le^x, α-fucosidase, α-galactosidase, β-galactosidase, rat development, forebrain, cerebellum**

Abbreviations: **Le^x, $Lewis^x$; SSEA-1, stage specific embryonic antigen-1; nLc_4, neolactotetraosylceramide; nLc_6, neolactohexaosylceramide; FT, fucosyltransferase; Lac, Lactose; LacNAc, *N*-acetyllatosamine; LNFI, lacto-*N*-fucopentaose I; NEM, *N*-ethylmaleimide; CNS, central nervous system**

Introduction

Glycoconjugates with Le^x epitopes containing the 3-fucosyl-*N*-acetyllactosamine, [Galβ1 → 4(Fucα1 → 3) GlcNAcβ1-R], and its sialylated form, sLe^x, [Neu5Acα2 → 3Galβ1 → 4(Fucα1 → 3)GlcNAcβ1-R] are present in various human and animal tissues, including the CNS. These epitopes occur on O- and N-glycoproteins, proteoglycans and glycolipids [1–7]. The Le^x epitope is known also as SSEA-1 (Stage Specific Embryonic Antigen-1), X, CD 15 (leuckocyte cluster of differentiation 15), FAL (3-α-fucosyl-*N*-acetyllactosamine), and TACA (tumor-associated carbohydrate antigen) [4, 6, 8]. These epitopes probably play a key role in cell–cell interactions during embryogenesis, including the development of the neuroaxis [4, 9–11]. sLe^x related structures are typically found as terminal residues of larger oligosaccharides on glycoproteins and glycolipids and are ligands for adhesion molecules, E- and P-selectins, which play key roles in leukocyte recruitment to sites of inflammation and tissue injury [9, 12, 13].

Information about expression of SSEA-1 glycoconjugates in developing CNS is comparatively limited. SSEA-1 glycolipids were initially shown to be stage- and region-specific antigens in developing embryonic rodent brain [14] and in postnatal human brain of a patient with fucosidosis [15]. These glycolipids (FucIII,nLc_4; FucV,nLc_6 and $[Fuc]_2$ III, V, nLc_6), are expressed in rat embryonic cerebral cortex at their highest levels during neurogenesis (at E15–E17 days) and decrease rapidly to negligible amounts soon after birth [16]. In the developing rat cerebellum the maximal expression of SSEA-1 glycolipid is seen during the period when external granule cells are migrating [17].

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Table 1. Structures described in this study

Lac	Ga1β1 → 4Glc
LacNAc	Galβ1 → 4G1cNAc
LNFPI	Fucα1 → 2Galβ1 → 3GlcNAcβ1 → 3Galβ1 → 4Glc
nLc_4	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Cer
$III^3FucnLc_4$	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Cer 3 \| Fucα1
nLc_6	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Ga1β1 → 4Glcβ1 → 1Cer
$III^3FucnLc_6$	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Ga1β1 → 4Glcβ1 → 1Cer 3 \| Fucα1
$V^3FucnLc_6$	Ga1β1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Cer 3 \| Fucα1
$III^3V^3FucnLc_6$	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Ga1β1 → 4Glcβ1 → 1Cer 3 3 \| \| Fucα1 Fucα1

Most glycoproteins and proteoglycans which express SSEA-1 antigens during brain development have not been identified [18]. Recently it has been demonstrated that FORSE-1, an antibody that labels regionally restricted subpopulations of progenitor cells in the embryonic central nervous system, recognizes the Le^x epitope on the proteoglycan phosphacan [19].

Since the final step in the biosynthesis of SSEA-1(Le^x) structures is catalysed by α1,3 fucosyltransferase(s) (α1,3FTs), this enzyme has a critical role in the expression of SSEA-antigens [20, 21]. In the last 5 years α1,3FTs have been intensively studied at the molecular level. Several cDNAs coding for various α1,3FTs have been isolated and characterized. These include FT III (Lewis type, on chromosome 19) with the broadest substrate specificity, FT IV (myeloid type on chromosome 11), FT V, FT VI (plasma type, chromosome 19) and FT VII (leukocyte type on chromosome 9) [2, 22–26]. In our laboratory we have used two complementary approaches to the study of these transferases in rat brain. One was cloning of rat α1,3FT [27]. The postulated amino acid sequence of this rat FT has 87% and 78% identity to the murine and human FT IV respectively, and is expressed in embryonic and adult rat brain. The second approach of direct characterization of the enzyme activities responsible for synthesis of SSEA-1 epitopes is presented in this paper. Since the expression of these antigens may be the result of a dynamic balance between the activities of FT and relevant glycosidases, we studied some of the hydrolases as well. None of the hydrolases have been fully characterized in isolated regions of the developing CNS. These glycosidases may be involved in degradation of either the precursors of SSEA-1 or the SSEA-1 epitope itself. In this report, we therefore, present our studies on FT, α-L-fucosidase, β-D-galactosidase and α-D-galactosidase in developing rat forebrain and cerebellum. We have also determined the expression of SSEA-1 glycolipids and glycoproteins during development in these brain regions. The data suggest region and substrate specific mechanisms affecting glycosyltransferases and glycosidases regulate the expression of SSEA-1 glycoconjugates during development. Preliminary results of these studies have been reported [28].

Materials and methods

Materials

GDP-[^{14}C]-Fucose (283 Ci mol^{-1}) was purchased from DuPont – New England Nuclear, *N*-Acetyllactosamine was obtained from Accurate Chemical Co., (Westbury, NY, USA). GDP-fucose, lactose, *N*-ethylmaleimide, CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), Triton CF-54 and taurodeoxycholate (sodium salt) were obtained from Sigma Chemical Co., Ltd (St Louis, MO, USA). Triton X-100 was purchased from Fisher Scientific Company (USA). Taurocholate (sodium salt) was obtained from Calbiochem (La Jolla, CA, USA). Paragloboside, nLc_4 was obtained from bovine red blood cells (RBC) according to [29]. Anion exchange resin (analytical grade) AG1-X8, 100–200 mesh, acetate form was obtained from BioRad. Pre-packed reversed-phase C_{18} cartridges (Bond-Elut C_{18}) and amino-function ion exchange cartridges with sorbent mass/column volume 100 mg per 1.0 ml column were obtained from Analytichem International (a division of

Varian). Protein assay reagents were obtained from Pierce. The fluorogenic substrates, 4-methylumbelliferyl derivatives of α-L-fucopyranoside, β-D-galactopyranoside and α-D-galactopyranoside for glycosidase assays were obtained from Sigma (St Louis, MO, USA). All other chemicals were obtained from commercial sources and were of the highest purity available (ACS or greater). For western blotting we used nitrocellulose membranes from BioRad, Super-Signal solution from Pierce, and Hyperfilm ECL for detection of chemiluminescence from Amersham.

Animals

Male and female Sprague-Dawley rats of different ages, kept on 12 h light/dark cycle with free access to food and water, were used in all experiments. Animals were killed by CO_2 asphyxiation, tissues removed and frozen at $-70\,^{\circ}C$ until use. The abbreviation 'P' is used to denote postnatal as in P1 for postnatal day 1.

Enzyme preparation

All enzyme procedures were performed on ice. Forebrain and cerebellum from Sprague-Dawley albino rats of various ages were used for enzyme assay. Tissues were homogenized in 20 mM MOPS/NaOH buffer (pH 7.4) containing Triton X-100 (0.5%) for ~ 1 min with an all glass homogenizer (Kontes Duall Tissue Grinder). Homogenates were centrifuged at $800 \times \mathbf{g}$ for 15 min and supernatants aliquoted (100 µl each) in Eppendorf tubes, frozen in liquid nitrogen and stored at $-80\,^{\circ}C$. Thawed extracts were used as the enzyme source on the day of fucosyltransferase assay.

Fucosyltransferase assays

Unless otherwise indicated, the standard incubation mixture (50 µl) contained 2.5 µmol of 3-(*N*-morpholino) propanesulfonic acid (MOPS)/NaOH buffer pH 7.4, 0.25 µmol of $MnCl_2$, 5 µmol of NaCl, 0.5 µmol of ATP, 104 nmol of *N*-acetyllactosamine (LacNAc), 0.5 nmol of GDP-L-[^{14}C]-fucose (which was diluted to a specific activity of $\sim 54\,000$ cpm nmol^{-1} with unlabeled GDP-fucose (Sigma), and 20 µl of tissue extracts (approximately 100–200 µg protein) as the enzyme source [27]. After incubation at 37 °C for 1–2 h, the reaction was terminated by the addition of 0.5 ml of cold water and the mixture was immediately applied to a 1 ml column of AG 1-X8 anion exchange resin (BioRad). The column was washed with 1 ml of water, and the combined aqueous effluents containing the fucosylated products were collected in scintillation vials and counted after addition of 4.5 ml of scintillation cocktail (Ready Safe, Beckman).

When nLc_4 (8–10 µg, unless otherwise noted), was used as the acceptor the assay mixture was sonicated for 1 min to allow the formation of glycolipid/detergent micelles. The enzymatic reaction was stopped by the addition of 0.5 ml of Theoretical Upper Phase (TUP) containing 0.1 M KCl mixture ($CHCl_3 : CH_3OH : H_2O$–60 : 960: 7.35 g KCl; TUP-KCl) and the mixture was applied to a pre-packed reversed phase C_{18} cartridge equilibrated with TUP-KCl mixture [30, 31]. The unreacted GDP-fucose was washed out with 1 ml TUP-KCl mixture followed by H_2O (3 ml). The lipid products were eluted with 2 ml of $CHCl_3 : CH_3OH$ (2 : 1) and 2 ml methanol. The lipid-containing eluates were combined, dried under a stream of nitrogen and radioactivity determined by scintillation counting in presence of 4.5 ml of Beckman ReadySafe scintillation cocktail. Values were corrected for fucose incorporation into endogenous acceptors. Fucosyltransferase specific activity was expressed in pmol of fucosylated product per mg of protein per h. The endogenous FT activity was not more than 5% of the total activity in presence of exogenous substrates.

Sensitivity of FT towards the inhibitor *N*-ethylmaleimide (NEM) was determined by preincubating the Triton X-100 extracts with 10 mM NEM for 1 h at 0 °C before the enzyme assay.

Identification of glycolipid products

Radioactive fucosylated enzyme products were generated with nLc_4 and a small amount of nLc_6 ($< 10\%$) in the presence of P17 rat cerebellar homogenates as source of FT (Figure 3). The amount of GDP-fucose in these samples was increased to 1 nmol ($\sim 54\,000$ cpm nmol^{-1}). Incubations were run for 2–15 h at 37 °C. The reaction was stopped by addition of 1 ml TUP-KCl, and the lipid product obtained as described above. The lipids were passed through an amino-function cation exchange column to separate neutral and acidic lipids. The neutral lipid fraction (65% of the total lipid radioactivity) was applied to an aluminum backed HPTLC plate and developed in chloroform : methanol : Aq. 0.25% $CaCl_2$ (50 : 40 : 10). The plate was dried and exposed to a Kodak X-O-Mat autoradiography film. After exposure, the HPTLC plate was developed for immunoblotting [32]. 7A antibody (kindly provided by Dr M. Yamamoto), that recognizes Galβ1 → 4[Fucα1 → 3]GlcNAc → R epitope, was used as the primary antibody followed by anti mouse HRP-conjugated IgM second antibody. Color was developed with 4-chloro-1-naphthol in presence of hydrogen peroxide. The precursors, nLc_4 and nLc_6, obtained from bovine RBC were identified by their reactivity to the mAb 1B2 (from Dr G. Schwarting, Shriver Center) that recognizes Galβ1 → 4GlcNAc → R epitope, and further confirmed by mass spectrometry.

Glycosidases assay

Activity of α-L-fucosidase, β-galactosidase and α-galactosidase were assayed with the fluorogenic substrates, 4-methylumbelliferyl α-L-fucopyranoside, β-D-galactopyranoside and -α-D-galactopyranoside respectively at pH 5.0 (fucosidase) or 4.5 (α- and β-galactosidases) at a final substrate concentration of 1 mM [33]. The reaction mixture

(100 µl) contained 20 µl of forebrain or cerebellar homogenate (approx. 80–100 µg of protein) and 80 µl of fluorogenic substrate dissolved in 0.1 M (citrate) 0.2 M (phosphate) buffer, pH 4.5 or 5.0. After 1 h of incubation at 37 °C the reaction was stopped with 2 ml of 0.25 M glycine-KOH buffer, pH 10.4. The fluorescence of enzymatically liberated 4-methylumbelliferone was determined in an Aminco SPF-500 C spectrofluorometer (Aminco Instruments) at an emission wavelength of 480 nm (excitation 365 nm). The specific activity of glycosidases was expressed as nmol of hydrolyzed substrate per mg protein.

Enzymatic reactions (fucosyltransferase and glycosidases) were linear with time, and with protein concentration within the parameters defined above.

Isoelectrofocusing

Forebrain (1.64 g) pooled from P2 and P3 rats was homogenized in a Potter-Elvehjem teflon-glass type homogenizer in 10 ml 1% Triton X-100 (v/v, Aq.) and centrifuged at $1000 \times \mathbf{g}$ to obtain the supernatant. The supernatant ($\sim$ 10 mg protein per ml) was diluted to 50 ml with distilled water and carrier ampholytes (pH 3–10, final concentration 1%), and separated by high-resolution preparative isoelectric focusing in a Rotofor apparatus (Bio-Rad). The run was completed in 4 h at 4 °C (with 12 W power) and the 20 resulting fractions collected. Ampholytes were removed and the samples concentrated by Macrosep 30 K ultrafiltration before determining the FT activity.

Glycolipid ELISA assay

Neutral glycolipids were extracted from cerebellum and forebrain (without the colliculus) at indicated ages [32], and used in ELISA assay as described [34]. SSEA-1 glycolipids from C57BL/6 mouse kidney [7] were used as the standard. SSEA-1 antibody (Dr G. Schwarting, Shriver Center) was the primary and HRP-conjugated anti-mouse IgM was the secondary antibody. Color was developed with o-phenylene-diamine in presence of hydrogen peroxide, reaction stopped with 0.1 M citric acid, and read at 490 nm in a Fisher Biotek 2000 Microkinetics ELISA reader.

Western blot analysis

Forebrain (without colliculus) and cerebellum were removed from rats at the indicated ages, and rapidly frozen on dry ice. The frozen tissue was partially thawed and homogenized using a Polytron-homogenizer in 25 mM Tris buffer pH 7.4, containing 0.32 M sucrose and 100 µg ml^{-1} phenylmethylsulfonyl fluoride. One hundred µg protein was boiled for 5 min in loading buffer (62.5 mM Tris, pH 7.0, 2% SDS, 10% glycerol, 0.00125% Bromphenol blue). Samples were run on 7.5% polyacrylamide gels and electrophoretically transferred to nitrocellulose (BioRad). Blots were stained with Ponceau S (Sigma) to ascertain equal amounts of protein were transferred in each lane. The membrane was incubated in 5% nonfat dry milk (Carnation) in Tris-buffered saline (TBS; 50 mM Tris pH 7.4, 150 mM NaCl) for 30 min at 22 °C, washed three times over 15 min in TBS containing 0.05% Tween-20 (TBST), followed by incubation in anti-SSEA-1 antibody (3B11; 10 µg ml^{-1}, a kind gift of Dr U. Drager, Shriver Center) in 5% milk in TBS overnight at 4 °C. The membrane was washed three times over 15 min in TBST and incubated in peroxidase-conjugated goat anti-mouse IgM antibody in TBS for 1h at 22 °C, then washed three more times (twice in TBST, then once in TBS, 15 min each). Signal was detected after incubation in Super-Signal TM Solution (Pierce) for 2–5 min and exposure to Hyperfilm ECL (Amersham) for variable lengths of time.

Other methods

Protein was determined in a 96 well microtiter plate with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Absorbance was read at 562 nm in a BT 2000 Microkinetics Reader Spectrophotometer (Fisher Biotech, Pittsburg, PA, USA). A standard curve was generated using a series of known concentrations of bovine serum albumin.

Results

FT in forebrain and cerebellum: differential activity

Our preliminary experiments on pooled forebrain from P2 and P3 rats showed that the FT activity was 10 times higher with LacNAc as acceptor than that obtained with lactose. In forebrain homogenates, the ratio of FT activity with LacNAc/Lac at P8–P9 was one half of that at P2–P3.

Determination of FT activity in fractions obtained after isoelectrofocusing (IEF) of Triton X-100 soluble forebrain extracts from young rats (P2 and P3) demonstrated the complexity of FT in nervous tissues. With LacNAc as acceptor, we see one major peak at pI 6.5 and a trailing fraction at pI 7.5 with lower activity (Figure 1A). However, with lactose and lactofucopentaose I as acceptors four components at pH 6.5, 7.8, 8.4 and 9.3 are seen, although the activity was much lower than that with LacNAc as acceptor (Figure 1B, C).

Some differences in substrate and region specificity of FT could be demonstrated even without isoelectrophoresis. For example in forebrain, with LacNac and Lac as substrates, the activity did not change from P17 to P19. In cerebellum with LacNAc as acceptor, FT activity at P19 was 50% less than that at P17; but with Lac as acceptor the activity remained unchanged. With nLc_4, in contrast, we observed a three-to-four-fold decrease in activity from P17 to P19 in both forebrain and cerebellum. These data point to the unique significance of each brain region, developmental age, and substrate specific regulation of FT activity that needed further investigation before a complete assessment of the role of this enzyme during development could be made.

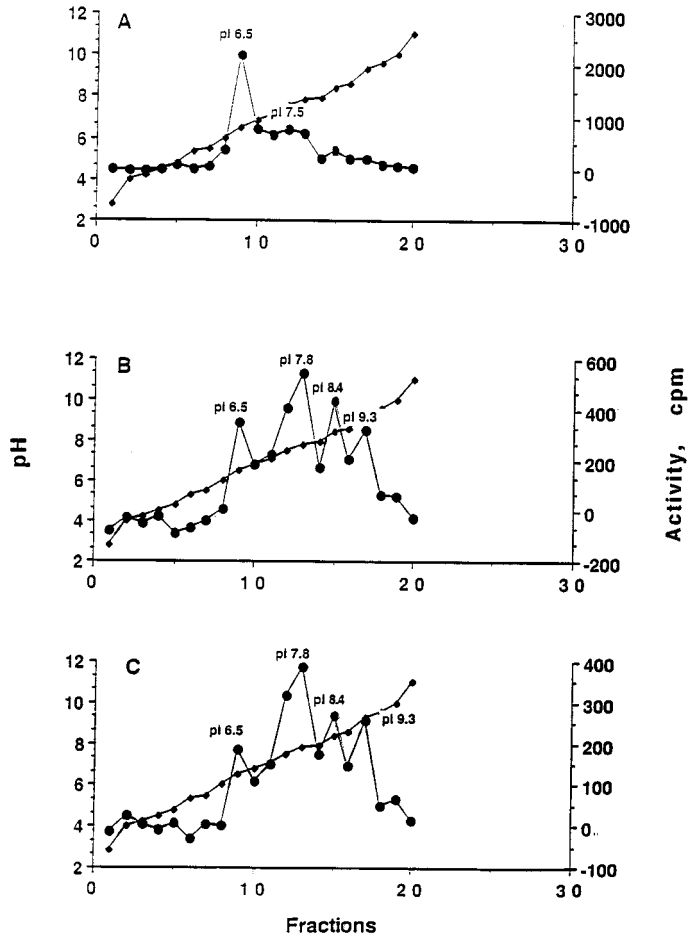

Figure 1. Isoelectrofocussing profile of *α*-fucosyltransferase activity from rat forebrain (P2–P3) at pH 3.5–10. **A**, **B**, **C**-LacNAc, Lac and LNFPI as acceptors, respectively. Closed circle, activity, closed square, pH. Values shown here and in subsequent figures represent means of duplicate determinations.

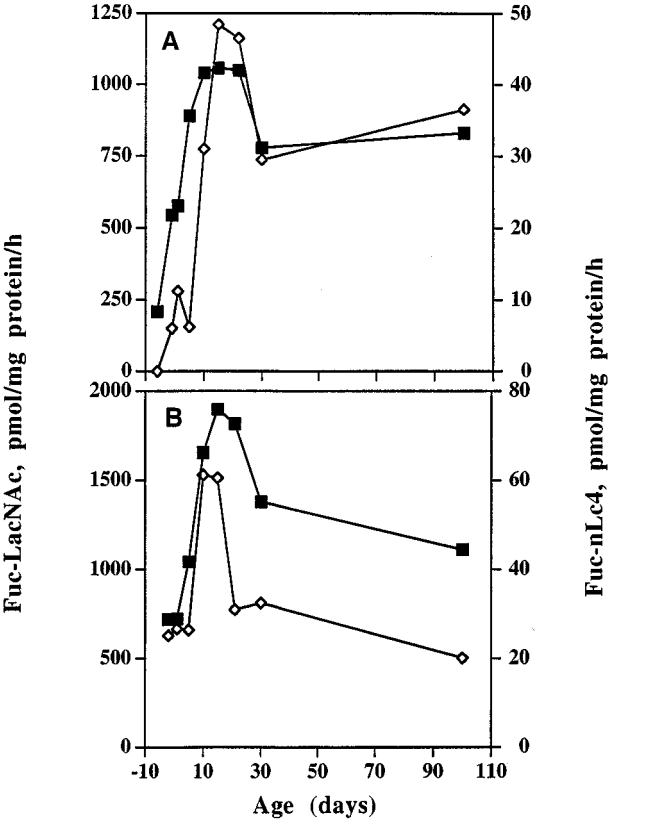

Figure 2. Developmental profiles of fucosyltransferase activity in rat brain with LacNAc (closed square) and nLc_4 (open diamond) as acceptors. (A) forebrain. (B) cerebellum.

Developmental profile of FT activity in forebrain and cerebellum

Temporal changes of the FT activity in forebrain and cerebellum between E15 through P100 are shown in Figure 2. In forebrain, maximal activity of FT was obtained at P15–P22 with both LacNAc and nLc_4. The enzyme activity was about 20-fold greater with LacNAc in comparison to that obtained with nLc_4 as acceptor (Figure 2A). In cerebellum, the activity of FT with nLc_4 was maximal at P10–P15. It decreased by 50% at P22, and decreased further at P100. However, with LacNAc as acceptor the maximal activity was obtained at P15 and decreased by approximately 50% at P100. At P15, the activity with LacNAc was 30-fold greater in comparison to that obtained with nLc_4 as acceptor (Figure 2B). The enzyme activity with nLc_4, decreased much more quickly in cerebellum than in forebrain. The developmental profile of FT activity in forebrain and cerebellum, therefore, demonstrates distinct tissue and substrate specificity.

Identification of glycolipid products

We examined enzymatic reaction products by HPTLC-autoradiography and immunostaining to elucidate substrate specificity of FT from rat cerebellum towards glycolipid acceptors, nLc_4 and nLc_6. The products from enzyme assays run for various times were examined. The autoradiogram showed two bands on HPTLC at all incubation time periods. Immunoblotting of the HPTLC plate with 7A antibody (that recognizes SSEA-1) demonstrated that both SSEA-1 positive bands, the faster moving major band (Fuc-nLc_4) and the slower moving minor band (Fuc-nLc_6), correspond to the bands seen on the autoradiogram (Figure 3). The two bands in lanes 1–4 of the autoradiogram correspond to SSEA-1 positive spots seen in lanes 1–4 of the immunoblot (Figure 3). The slow moving minor band on the autoradiogram was visible on the immunoblot after the 2 h assay time point. Longer incubations, apparently, resulted in sufficient amounts of fucosylated products to be detectable by the antibody. On the basis of the relative mobility

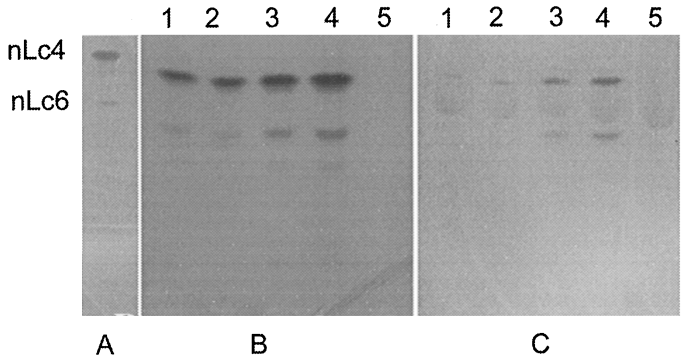

Figure 3. Idenification of glycolipid precursors, and glycolipid products of *a*1,3-fucosyltransferase reaction on HPTLC. (A) Immunoblot with 1B2 antibody reactivity of precursor (nLc_4, nLc_6) glycolipids from bovine red blood cells. (B) Autoradiogram of glycolipid products, separated on HPTLC-plate, from cerebellar fucosyltransferase enzyme reaction. Lanes 1–4 are neutral glycolipid products obtained after 2, 4, 15, and 15 hours enzymatic reaction respectively. Lane 5 is a blank incubation without exogenous precursors. (C) Immunoblot (7A-reacting antibody) of the HPTLC-plate from B.

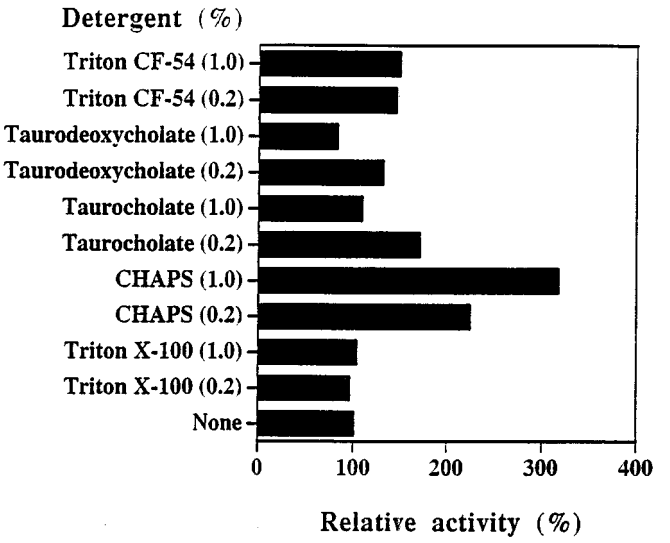

Figure 4. Effect of different detergents on nLc_4 fucosyltransferase activity in rat cerebellum (17 day old). All assay samples contained Triton X-100 (0.2%) as basal level of detergent.

and immunoreactivity, the upper and lower immunoreactive bands probably represent Fuc-nLc_4 and Fuc-nLc_6 respectively. A third band migrating below Fuc-nLc_6 on the autoradiogram, in the 15 h assay, may be the difucosylated derivative of nLc_6. These data indicate that the fucosylated enzymatic products obtained with nLc_4 and nLc_6 are SSEA-1 positive and therefore have structures with fucose α1,3 linked to *N*-acetylglucosamine.

Effect of *N*-ethylmaleimide (NEM) on FT activity

To identify the type of α1,3 FT in developing brain, we investigated the effect of one of the sulfhydryl reagents, NEM, on FT activity with LacNAc. In forebrain at E15, E20, P15, P17 and P22 NEM had negligible effect on enzyme activity. Similarly in cerebellum at P17, NEM had no significant effect on FT activity.

Effects of detergents on nLc_4 FT activity

Figure 4 shows the effect of different detergents on FT activity in P17 cerebellum with nLc_4 as acceptor. The assay mixtures also contained 0.2% Triton X-100 (see Materials and methods) in addition to the different detergents tested. Under these conditions highest activity of FT was obtained in presence of 1% CHAPS. It was more than three-fold higher than that with 1% Triton X-100. The enzyme activity was lower with 1% taurocholate and taurodeoxycholate than that obtained with 0.2% of either detergents. Triton CF-54 at 0.2 or 1.0% activity by approximately 50% over the value obtained without any detergent.

Glycosidase activity

In forebrain during development, α-L-fucosidase activity was highest at E15 and decreased steadily thereafter until P100. In cerebellum the activity of fucosidase was highest at P10, and decreased by 3-fold by P100 (Figure 5A).

The developmental pattern of β-D-galactosidase and α-D-galactosidase activity was different in forebrain and cerebellum (Figure 5). In forebrain, the activity profile of these enzymes was biphasic. It was high at E15 and decreased by almost one half by P5. The activity then increased by one and a half fold at P15 (β-galactosidase) and P22 (α-galactosidase). In the adult these glycosidases were significantly active (Figure 5B).

In cerebellum, the activity of β-D-galactosidase was low before birth and increased almost two-fold between E20 and P21, and then decreased slightly at P30 through P100 (Figure 5C). Both hydrolytic enzymes had significant activity in the adult. It is interesting to note that the developmental expression of fucosidase is strikingly different from the other two aforementioned glycosidases in cerebellum.

Expression of Lewis^x glycoconjugates

We examined the levels of SSEA-1 glycoconjugates in developing rat forebrain and cerebellum during development to determine the levels of the final endogenous products of the FT reactions. Glycolipid and glycoprotein determinations were performed on tissues obtained from the same rat litters. Our results (Figure 6A) show a peak of SSEA-1 glycolipid detected in both tissues during early postnatal development within the region of high fucosyltransferase enzyme activity. The pattern seen for SSEA-1 glycoproteins differs from that seen for glycolipids in both forebrain and cerebellum. In forebrain, the major glycoprotein had a molecular weight greater than 220 kDa, and was present in comparable amounts at all ages from P1 to P21 (Figure 6B.

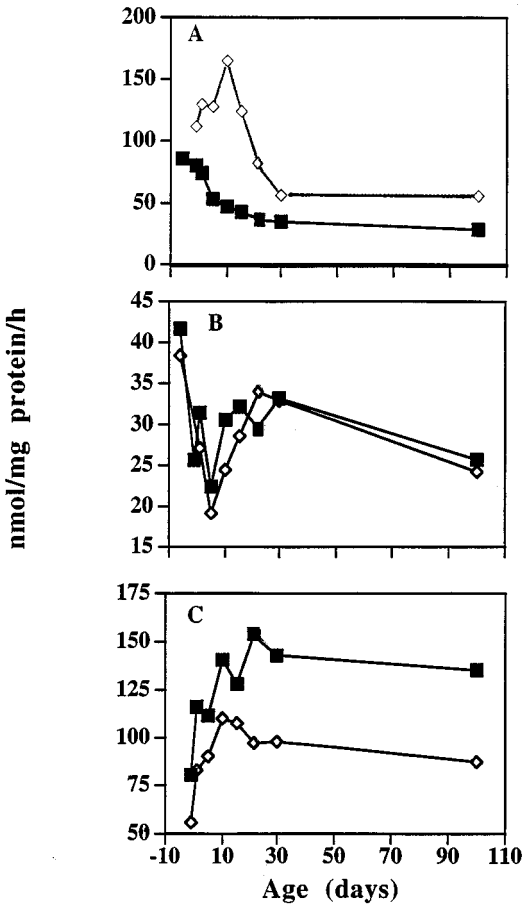

Figure 5. Profile of glycosidase activities in rat brain during development. (A) fucosidase activity in forebrain (closed square) and in cerebellum (open diamond); (B) profile of β-D-galactosidase (closed square) and α-D-galactosidase (open diamond) activities in rat forebrain (B) and cerebellum (C).

It should be noted that Ponceau S staining indicated that lower amounts of protein were transferred in lanes containing forebrain samples for P4 and P21; data not shown). In cerebellum, in addition to this high-molecular-weight-glycoprotein, another band of about 97 kDa also was detected in essentially constant amounts at all ages from P1 to P21 (Figure 6B). Additional glycoproteins of intermediate size were observed in cerebellum that appeared around P4 and were expressed in comparable amounts through P21. These results indicate differential regulation of SSEA-1 containing glycolipids and glycoproteins through development. Similar results have been obtained in the mouse (data not shown).

Discussion

Since SSEA-1 glycoconjugates are expressed perinatally and are probably involved in cellular migration events during brain development, we studied both the biosynthetic and degradative enzymes that in balance may be the critical elements in developmental expression of these glycoconjugates. Further the data were obtained in isolated brain regions of forebrain and cerebellum, since their development is temporally distinct and leads to architecturally different structures.

In forebrain, FT activity was maximal at P15–P22 with either LacNAc or nLc_4 as acceptors. However, in cerebellum the maximal activity with LacNAc was obtained at P15, and between P5–P10, with nLc_4. Adult tissues had significant activity with both substrates. Identification of glycolipid products of the enzymatic reaction by the monoclonal antibody 7A, showed that the compounds obtained with nLc_4 and nLc_6 as acceptors had α1,3 fucosylated *N*-acetylglucosamine structures (SSEA-1 epitope). Our observations and those of Chou *et al.* [16] indicate that the SSEA-1 epitope in developing brain is expressed as fucosylated nLc_4 ($III^3FucnLc_4$), nLc_6 ($III^3FucnLc_6$, $V^3FucnLc_6$), and possibly also as difucosylated nLc_6 ($III^3V^3FucnLc_6$). The maximal activity of FT with lipid acceptors corresponds to the period of maximal expression of SSEA-1 glycolipids in the cerebellum ([16], our results). This correspondence of lipid FT activity and epitope expression on lipids is consistent with a regulatory role of α1,3 FT in the expression of SSEA-1. However, the low amounts of SSEA-1 glycolipids in adult cerebellum, in the presence of increasing amounts of precursor [16], and the high FT activity in presence of LacNAc and nLc_4 in adult cerebellum, suggests that this enzyme alone may not be regulating the expression of SSEA-1 glycolipids. In forebrain as well, the high FT activity in the adult also contrasts with the minimal expression of SSEA-1 glycolipid ([16], our results), although in this case this discrepancy is apparently due to decreasing levels of precursor [16].

The regulation of SSEA-1 glycoproteins has been less well studied than that of SSEA-1 glycolipids: they are not all identified, and the levels of their precursors are unknown. The high molecular weight ($>$ 220 kDa) glycoprotein which we detected in forebrain and cerebellum is probably the previously identified proteoglycan phosphocan [19], and is present in virtually unvarying amounts in both postnatal forebrain and cerebellum. Several other SSEA-1 glycoproteins also were detected in cerebellum. However, there is no obvious correlation between changes in FT activity and changes in SSEA-1 glycoproteins. This supports the suggestion that different fucosyltransferase activities may be involved in fucosylating glycolipid and glycoprotein substrates in the brain.

We did not use a glycoprotein acceptor in these studies. Previously, in rat cerebral hemispheres, the enzymatic activity of GDP-fucose: asialofetuin fucosyltransferase has been shown to increase after 3 days of birth to a maximum at 21 days [25]. These data with the glycoprotein acceptor show some similarity to our data on FT activity, in that they show a postnatal increase in FT activity. However, they differ in that we do not observe the biphasic maxima seen with

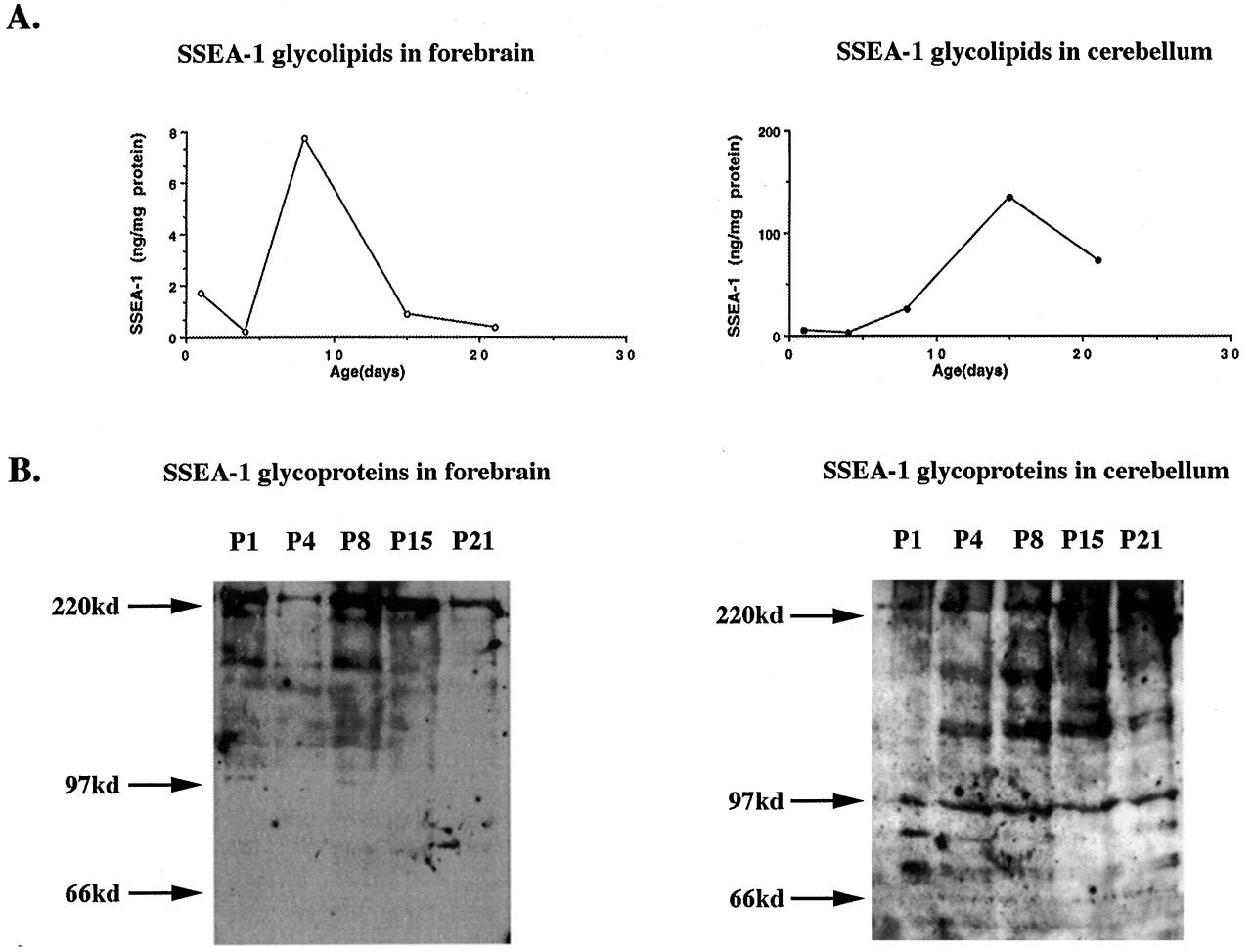

Figure 6. Glycoconjugate expression during development in cerebellum and forebrain: (A) Glycolipids (B) Glycoproteins. Ponceau S staining of the blots indicated that lower amounts of protein were transferred in lanes containing forebrain samples at P4 and P21.

asialofetuin. Thus, although broad similarities in enzymatic activity profiles are seen with various lipid, oligosaccharide and protein substrates, both within and between regions, clear differences are seen. The observed region specific differences in enzyme activity towards different acceptors may be due to different subtypes of α1,3FT or due to different microenvironments presented within these tissues. The presence of region specific inhibitors [34] or activators of FT also might be considered. Additionally, it is not clear whether the α1,3 FT(s), studied here with Lac, LacNAc and nLc_4 acceptors are responsible for fucosylation of every kind of acceptor *in vivo*. It is quite possible that different regions of rat brain express various types or subtypes of FTs, with different substrate specificity, not only for carbohydrate recognition sites (CARS-enzymes), but also for the aglycon part of the glycoconjugate acceptors, glycoproteins and/or glycolipids [36, 37].

Isoelectrofocussing of Triton-X100 extracts of forebrain of young rats (P2 and P3) provides further evidence for the presence of multiple FTs in rat brain. The FT activity with LacNAc eluted at pI of 6.5 as a major peak followed by a minor peak, and four separate activities of FT were observed with lactose or lactofucopentaose I (LNFP I) at pIs of 6.5, 7.8, 8.4 and 9.3. The activity of FT with LacNAc was higher than with the other acceptors. The two acceptors, Lac and LNFPI, could act as substrates for α1,3/4 FTs. In the presence of Lac as acceptor, Fucα1,3Glc linkage (3-fucosyllactose) might be formed, and with LNFPI (oligosaccharide type I chain) as acceptor two distinct possibilities of fucosylation can be suggested: Fucα1,3 linkage to Glc, and Fucα1,4 linkage to GlcNAc. Taken together, these data suggest the presence FT IV- and FT III-activities in perinatal rat brain. The ability to fucosylate LNFPI is in contrast to that recently reported for bovine enzyme FT III-V-VI [38].

Several distinct types of mammalian α1,3 FTs (and subtypes of α1,3 FT) with peculiarities in substrate specificity and properties have been identified [39–42]. One of the features that clearly distinguishes different subtypes of α1,3 FT(s) is inhibition by the protein sulfhydryl group

modifying reagent, *N*-ethylmaleimide (NEM) [43]. FT IV-type enzymes are not inhibited by this reagent. Experiments with LacNAc acceptor showed that 10 mM NEM had no effect on FT activities in forebrain and cerebellum. Our recent studies on a cloned FT from rat cerebellum suggest that the resistance to NEM may be due to the substitution of cysteine by threonine in the GDP-fucose binding domain [28] similar to that seen in human FT IV and FT VII, in contrast to the NEM sensitivity demonstrated by FT III, V and VI that all have a cysteine residue in the GDP-fucose binding domain [44]. Since the activity of FT is highest with LacNAc in all regions and this activity is also insensitive to NEM, we postulate that the major type of α1,3 FT(s) in rat brain is a FT IV-like enzyme. Further, its ability to fucosylate nLc_4 lends further credence to this suggestion, and distinguishes it from the FT VII-like enzyme that only accepts sialylated acceptors. Recently NEM insensitivity to inhibition was also shown for α1,3 FT from developing human myeloid cells [43].

Rat α1,3FT was differentially activated by various detergents. Fucosylation of nLc_4 by cerebellar FT was increased three-fold by 1% CHAPS added to incubation medium that already contained 0.2% Triton X-100 from the tissue homogenate. Taurocholate, taurodeoxycholate and Triton CF-54 (0.2%) also stimulated FT activity 70%, 30%, and 50% respectively. Since phospholipids, fatty acids and other membrane components may markedly influence enzyme activity by different mechanisms [45, 46], the effect of detergents on FT activity might reflect an *in vivo* sensitivity to the lipid environment of the enzyme, with a significant impact on enzyme activity and on the nature of the products formed, and hence the expression of different cell surface antigens [41].

Data concerning the role of glycosidases in expression of important brain glycolipid antigens during embryogenesis and postnatal development are very limited [47, 48]. To determine expression of some of the glycosidases which might modify the SSEA-1 antigen or potential precursors, we studied the developmental profile of α-L-fucosidase, β-D-galactosidase and α-D-galactosidase. The first two enzymes might be involved in hydrolytic modifications of SSEA-1 antigens. α-D-galactosidase activity may be important in modulating the expression of fucosylated tetrasaccharides based on a Galα1,3Galβ1,4GlcNAc core structure (fucosylated by an α1,3 FT). Such an epitope as a potential precursor to SSEA-1 has been described [2, 49].

In general, the maximal activity of different glycosidases coincided with the period when the activity of α1,3 FT in forebrain and cerebellum was also maximal. It is therefore possible that expression of SSEA-1 antigens is a result of a dynamic balance between endogenous glycosyltransferase and glycosidase activities during development. Such a possibility has been demonstrated for expression of Gb_3, identified as the mammalian cell membrane glycolipid ligand for Shiga and Shiga-like toxins. Incubation of toxin-resistant HeLa T5 cells with a specific competitive inhibitor of lysosomal α-galactosidase, 1,5-dideoxy-1,5-imino-D-galactitol (DIG) increased the cellular content of Gb_3 by two-fold, changed the cellular phenotype, increased the binding of Shiga toxin to cells, and increased cellular sensitivity to toxin [50]. Further, we have observed a decrease in SSEA-1 glycolipids and a concomitant increase in precursor paraglobosides in cerebellum of a sheep model of GM1 gangliosidosis during development, presumably due to an increase in fucosidase activity (Koul and McCluer, unpublished observations).

The role of fucosidases in the expression of fucosylated glycoconjugates may be larger than what has been accorded them so far. Mammalian α-fucosidases hydrolyze a wide variety of fucose-containing compounds, including milk oligosaccharides [51], fragments of blood group A and H substances [52], H antigen glycolipids [53] and a synthetic fluorogenic lipid-like fucoside, 6-hexadecanoylamino-4-methylumbelliferyl α-L-fucopyranoside [33]. Some types of α-fucosidases associated with microsomal membranes from human brain are capable of hydrolyzing hydrophobic fucosylglycoconjugates at pH 3.4 without any activator proteins or detergents [54]. Further investigation on aforementioned glycosidases may help to clarify the role of these enzymes in modifications of SSEA-1 glycoconjugates.

Dasgupta *et al.* [55] have recently reported on expression of FT and α-fucosidase in whole brain homogenates of rat. Their data show two peaks of activity for FT at E18 and P15, and two peaks of activity for α-fucosidase at E21 and P5. Our data reported here, in contrast, were obtained with isolated brain regions (forebrain and cerebellum). Because these regions are distinct in their cytoarchitecture and development, we believe that it is important also to study the FT activities of these regions during development, their substrate specificities, and isoelectrofocusing profiles, separately.

In conclusion, our data demonstrate that rat forebrain and cerebellum express predominantly a FT IV-like α1,3 FT activity, in addition to a lower level of α3/4 FT type enzyme(s). The α1,3 FT enzyme(s) is resistant to NEM and capable of fucosylating hydrophilic LacNAc and hydrophobic nLc_4 acceptors. The developmental profile with oligosaccharide and lipid acceptors was specific for tissue and substrate. The profiles of FT, glycosidases, and SSEA-1 glycolipids and glycoproteins in forebrain and cerebellum during development suggest that the expression of the SSEA-1 glycoconjugates may be the result of a dynamic balance between the synthetic and hydrolytic enzymes. Further, the balance and mix of FTs may be different in cerebellum and forebrain resulting in different profiles of expression of the SSEA-1 glycoconjugates.

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