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Biosynthesis *in vitro* of neolactotetraosylceramide by a galactosyltransferase from mouse T-lymphoma: purification and kinetic studies; synthesis of neolacto and polylactosamine core

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The galactosyltransferase, GalT-4, which catalyses the biosynthesis *in vitro* of neolactotetraosylceramide, nLcOse4Cer (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer) from lactotriaosylceramide, LcOse3Cer (GlcNAcβ1-3Galβ1-4Glc-Cer), and UDP-galactose has been purified 107 500-fold from a mineral oil induced mouse T-lyphoma P-1798, using affinity columns. The purified enzyme is partially stabilized in the presence of phospholipid liposomes. Two closely migrating protein bands of apparent molecular weights 56 kDa and 63 kDa were observed after sodium dodecyl sulfate polyacrylamide gel electrophoresis of highly purified mouse GalT-4. These two protein bands, when subjected to limited proteolysis, resulted in three peptides with identical mobilities indicating amino acid sequence identity between the proteins. Both protein bands from P-1798 gave a positive immunostain when tested with polyclonal antibody against bovine lactose synthetase (UDP-Gal:Glc β4-galactosyltransferase) following Western blot analysis on nitrocellulose paper. The enzyme has a pH optimum between 6.5 and 7.0 and like all other galactosyltransferases, GalT-4 has absolute requirements for divalent cation (Mn^{2+}). The K_m values for the substrate LcOse3Cer and donor UDP-galactose are 110 and 250 μM, respectively. Substrate competition studies with LcOse3Cer and either asialo-agalacto-α1-acid glycoprotein or *N*-acetylglucosamine revealed that these reactions might be catalysed by the same protein. The only other glycolipid which showed acceptor activity toward the purified GalT-4 was iLcOse5Cer (GlcNAcβ1–1-3Galβ1-4Lc3), the precursor for polylactosamine antigens. However, competition studies with these two active substrates using the most purified enzyme fraction, revealed that these two reactions might be catalysed by two different proteins since the experimental values were closer to the theoretical values calculated for two enzymes. Interestingly however, it seems that the GalT-4 from P-1798 has an absolute requirement for an *N*-acetylglucosamine residue in the substrate since the lyso-derivative ($GlcNH_2$β1-3Galβ1-4Glc-sphingosine) of the acceptor glycolipid LcOse3Cer is completely inactive as substrate while the K_m and V_{max} of the reacetylated substrate (GlcNAcβ1-3Galβ1-4Glc-acetylsphingosine) was comparable with LcOse3Cer. Autoradiography of the radioactive product formed by purified P-1798 GalT-4 confirmed the presence of nLcOse4Cer, as the product cochromatographed with authentic glycolipid. The monoclonal antibody IB-2, specific for nLcOse4Cer, also produced a positive immunostained band on TLC as well as giving a positive ELISA when tested with radioactive product obtained using a highly purified enzyme from mouse P-1798 T-lymphoma.

Keywords: galactosyltransferase, glycolipid biosynthesis, neolacto-series, tumour antigens

Abbreviations: EDTA, ethylenediamine tetraacetate; ME, β-mercaptoethanol; PEG, polyethylene glycol; PBS, phosphate buffered saline; Suc, sucrose; Mn^{2+}, manganese; Gal, galactose; GlcNAc, *N*-acetylglucosamine; UDP-Gal, Uridine diphosphate galactose; Ab, antibody; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; ECB, embryonic chicken brain; Cer, ceramide; nLc4 or nLcOse4Cer; Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer, neoLactotetraosylceramide; Lc3 or LcOse3Cer, GlcNacβ1-3Galβ1-4Glc-Cer, lactotriaosylceramide; iLc5, iLcOse5Cer, GlcNAcβ1-3nLcOse4Cer; nLc6, nLcOse6Cer, Galβ1-4iLcOse5Cer; $SA^{-}Gal^{-}\alpha_1AGP$, asialo-agalacto-α1-acid glycoprotein; TLC, thin layer chromatography.

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Introduction

Glycosphingolipids (GSLs) are integral parts of the membrane components which play a major role in different cellular events including cell-cell adhesion and cell-cell recognition [1]. The changes in GSL compositions during tumorigenesis are quite well documented in the literature [2]. The notion of over-producton of fucosylated- (Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-Cer; Le^x) and sialylated-fucosylated (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-Cer; SA-Le^x) GSLs, as well as of their precursor neolactotetraosylceramide (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer; nLcOse4Cer) during tumour progression, has recently become well accepted [3]. The synthesis of the core neolactotetraosylceramide of the type-2 lactoseries glycosphingolipid is therefore a major event in embryonic development, during differentiation and during tumorigenesis in various eukaryotic systems.

The galactosyltransferase, GalT-4 [UDP-Gal: Lc3 (GLcNAcβ1-3Galβ1-4Glc-Cer) β1-4Galactosyltransferase] which catalyses biosynthesis *in vitro* of nLcOse4Cer (Galβ1-4Lc3), the most common precursor of all type-2 ABH blood-group, polylactosamine-antigens, and, most importantly, of tumour antigens, was first characterized in rabbit bone marrow [4]. UDP-Gal:GlcNAc-R β4-galactosyltransferase (EC 2.4.1.38; 2.4.1.90) is widely distributed in eukaryotic systems which catalyses the formation of a commonly occurring glycosidic linkage, Galβ1-4GlcNAc-R, present in both glycoprotein and glycolipid antigens [5–10]. Lactose synthetase (EC 2.4.1.22) is a non-covalent complex of UDP-Gal:GlcNAc-R β4-galactosyltransferase and α-lactalbumin and catalyses the formation of lactose in the mammary gland. UDP-Gal:GlcNAc-R β4-galactosyltransferase has been studied in great detail; the enzyme has been purified from many sources and kinetic parameters have been established [7–10]. GalT-4 has many properties that are identical to those of UDP-Gal:GlcNAc-R β4-galactosyltransferase, and the two enzyme activities are catalysed by the same protein. GalT-4 has been studied in various normal and tumour sources [4–6, 11–17]. GalT-4 does not require the presence of α-lactalbumin for activity but has an α-lactalbumin-binding site since it binds to the α-lactalbumin-sepharose 4B affinity columns. However, a galactosyltransferase (UDP-Gal:Lc3 β1-4galactosyltransferase) has been reported from rainbow trout which does not have an α-lactalbumin binding site [18].

The genes for UDP-Gal:GlcNAc-R β4-galactosyltransferase from various sources (bovine, murine, human) have been cloned, and complete DNA sequences have been reported [19–24]. Recently, a partial cDNA clone of GalT-4 has been isolated from embryonic chicken brain and has been cloned and expressed as an active protein by our laboratory [14, 25, 26]. Cloning and sequencing of different glycosyltransferases from various sources [27–32] revealed that very little sequence homology occurs between glycosyltransferases although there is a similarity in the protein domain structures of these enzymes. All the glycosyltransferases cloned so far have been characterized as having a similar domain structure with a short N-terminal peptide and a comparatively longer carboxy-terminal peptide which is preceded by a putative transmembrane sequence [33]. It is believed that the carboxy-terminal peptide faces the luminal side of the Golgi-membrane. We report here the purification of the galactosyltransferase GalT-4 from mineral oil induced mouse T-lymphoma. The purified enzyme exhibited two closely migrating protein bands at 56 kDa and 63 kDa, both of which are immunostained with anti-embryonic chicken brain as well as anti bovine galactosyltransferase antibodies. Limited proteolysis of these protein bands resulted in the production of three peptides of similar mobilities indicating an amino acid sequence similarity between the proteins. The reaction catalysed by the mouse tumour galactosyltransferase is given below:

$$\text{UDP-[}^3\text{H]Galactose} + \text{GlcNAc}\beta1\text{-}3\text{Gal}\beta1\text{-}4\text{Glc-cer} \rightarrow$$

$$\text{Gal}\beta1\text{-}4\text{GlcNAc}\beta1\text{-}3\text{Gal}\beta1\text{-}4\text{Glc-cer}$$

Materials and methods

MATERIALS

UDP-[^{3}H]Gal and unlabeled UDP-Gal were obtained from American Radiolabeled Company and Sigma Chemical Company, respectively. The following materials were obtained from other commercial sources: Triton CF-54, taurodeoxycholate, α-lactalbumin (bovine milk), cyanogen bromide, UDP-hexanolamine, sepharose 4B, bovine β4-GT (Sigma Chemical Co.); SG-81 and Whatman 3MM papers (Scientific Products); silica gel G and TLC plates (Brinkman Instruments). α-lactalbumin-sepharose 4B and UDP-hexanolamine-sepharose 4B were prepared following the published methods [34, 35]. Lactotriaosylceramide (LcOse3Cer) acceptor and other glycosphingolipids used in this study were prepared from bovine and rabbit erythrocytes using our previously published methods [5, 6, 12]. Clam and papaya glycosidases used for the preparation of the substrates as well as for the cleavage of the radioactive products were prepared in our laboratory [36]. Antibody against embryonic chicken brain GalT-4 and bovine β4GT were produced in rabbit in our laboratory [14]. Monoclonal antibodies IB-2 and HMST-1 were gift samples were Dr Iwamori of Tokyo University, Japan.

PREPARATION OF RE-ACETYLATED LYSO-GLYCOLIPIDS

Lyso-glycosphingolipids (deacetylatedoligosaccharide-sphingosine) were prepared with slight modification of our previously published method [37, 38]. Glycolipids were hydrazinolyzed with hydrazine in the presence of a

catalytic amount of hydrazine sulfate at 150 °C for 20 h. Both sphingoid amines and free amine groups in the oligosaccharide portion of the glycolipid ($GlcNH_2$, $GalNH_2$ and $NeuNH_2$) were then reacetylated with acetic anhydride in the presence of triethylamine [37]. Reacetylated lyso-GSLs thus prepared were devoid of fatty acids in the polar head groups [oligoglycosyl-(acetyl-)sphingosine] and acted as suitable acceptors for studying the requirement for hydrophobic moieties for any glycolipid:-glycosyltransferases [39].

PRODUCTION OF MOUSE TUMOURS

BALB/C mice (two or four, 6–8 weeks old) of Andervont origin (An) were first primed with pristane at least 10 days (maximum 60 days) before injection of minced tumour intraperitonially. We received minced frozen tumours of different origins (P-1798, ABLS-140) from Hazleton Laboratory America Inc. (Rockville, MD). Once the tumour growth was apparent by enlarged abdomen in the recovery mice, the ascites fluid was collected by puncturing the abdomen with a 16 gauge, 4 cm sterile needle just interior to the inguinal region. Aliquots (0.3–0.4 ml) of the ascites fluid were then injected subcutaneously into the flanks of these mice 1 cm above the inguinal area up to the auxiliary region, being careful not to puncture the peritoneum, and into another set of mice (15–20) of the same strain with a 22 gauge 4 cm needle. The tumours were collected once they reached the desired size (1–2 cm) as indicated by the appearance of the mice (3–4 weeks) after sacrificing the animals using carbon dioxide. We have been able to grow the above-mentioned tumours in substantial quantities (30–60 g per 30 days; 3–4 g tumour tissue per mouse) for our enzymatic work.

PURIFICATION OF ENZYMES

All steps were carried out at 4 °C. The following buffers were used: Buffer A: 0.32 M sucrose containing 20 mM HEPES (pH 7.0), 1 mM EDTA, and 0.014 mM β-ME; Buffer B: 20 mM HEPES (pH 7.0), 0.1% 8K polyethylene glycol (PEG), 0.014 mM β-ME, 0.02% Triton CF-54, 0.5 M NaCl, 25 mM GlcNAc and 25 mM $MnCl_2$; Buffer C: Buffer B minus GlcNAc and $MnCl_2$; Buffer D; Buffer C plus 25 mM EDTA.

Step 1

The tissues were homogenized in four volumes of Buffer A using a polytron 10ST homogenizer. The crude homogenate was centrifuged at 12 000 rpm for 30 min using a Beckman JA-14 rotor, and the resulting residue was homogenized in two or three volumes of buffer A.

Step 2

The galactosyltransferase was extracted from the residue at a protein to detergent ratio of 2 for at least 1 h using 0.4% Triton CF-54 in the homogenizing buffer. The extracted residue was then centrifuged at 105 000 × **g** for 45 min in a Beckman Ultracentrifuge (L8-M). Approximately 80–90% of the GalT-4 activity has been solubilized from mouse tumour homogenate.

Step 3

The detergent soluble supernatant (DSS) containing GalT-4 was purified using an α-lactalbumin-sepharose 4B affinity column. The column (1 × 10 cm) was first equilibrated by washing with buffer B, and the DSS was loaded on the column after adjusting to 25 mM each of $MnCl_2$ and GlcNAc. The column was washed thoroughly with buffer B (10 column volumes) until the protein values of the wash fraction become negligible. The bound GalT was then eluted by washing the column with buffer C, and eluents were collected in fractions (10 × 6–7 ml). The enzymatically active fractions in the eluent were combined (El.1) and both Mn^{2+} and GlcNAc (25 mM each) were added for stability of the enzyme.

Step 4

The combined eluent El.1 from the first alpha-lactalbumin column was passed through a second alpha-lactalbumin column following exactly the same procedure as described for step 3 with the exception that a smaller column (1 × 3 cm was used followed by a collection of smaller volumes of eluted fractions (2–3 ml each). The combined enzymatically active eluted fraction (El.2) was again adjusted to 25 mM Mn^{2+} and 25 mM GlcNAc.

Step 5
UDP-hexanolamine-sepharose column chromatography was used as the final step of purification

One ml UDP-hexanolamine-sepharose 4B column was equilibrated with buffer B before loading the El.2 fraction. The column was washed with 2 column volumes of buffer B after which the bound GalT-4 was eluted with buffer D. Fractions (0.5 ml) were assayed for GalT-4 activity, and the enzymatically active fractions were combined. The EDTA was dialysed out, and GlcNAc and Mn^{2+} were added to the pooled fraction. The enzyme becomes very unstable at this stage of purification due to extremely low protein concentration and was stable only for 2 or 3 days.

PROTEIN ESTIMATION

Either the Lowry [40] or Biorad method [41] was used for protein determination. For the most pure fraction, the detergent was removed by extensive dialysis after dilution under its critical micellar concentration, followed by lyophilization, before estimation of the protein.

SDS-PAGE ELECTROPHORESIS

The concentrated purified proteins, after removal of detergents, were subjected to 10% polyacrylamide gel electrophoresis according to Laemmli [42] with molecular

weight standards. The protein bands were then visualized by silver staining [43].

PROTEIN FINGERPRINTING

Protein fingerprinting involves partial proteolytic digestion of the purified proteins followed by SDS-PAGE separation of the peptide fragments. It generates a fingerprint that is characteristic of the particular protein substrate and the protease used for cleavage [44]. The degree of similarity in the peptide fingerprint of two proteins can be correlated with the degree of similarity between the proteins at the amino acid sequence level.

One hundred μl of purified mouse lymphoma GalT-4 (5–10 μg protein) were first run on an 8%, 1.0 mm thick mini gel for SDS-PAGE. The gel was stained for 20 min with Coomassie blue stain (0.25% Coomassie brilliant blue in 25% isopropanol and 7% glacial acetic acid) followed by destaining in 5% acetic acid for a defined time of less than 2 h [45]. Two visualized protein bands were excised from the gel and the gel slices were carefully loaded onto a second 1.5 mm thick minigel with 16% separating and 4.5% stacking gels. The gel slices were then overlaid with the overlay solution (0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 3% ME, 20% glycerol, and 0.005% Bromophenol blue). The *Staphylococcus aureus* V8 protease was dissolved in reaction buffer, diluted with 0.025% Bromophenol blue containing 50% glycerol, and loaded over the gel slices in the wells (5 μl). The electrophoresis was run at 70 V until the dye bands migrated to the interface between the stacking and separating gels. The electrophoresis was stopped at this point, and proteolytic digestion was allowed to run for 30 min at room temperature. The resulting peptides were finally resolved on the separating gel by electrophoresis at 200 V and were visualized following silver staining protocol [43].

IMMUNOSTAIN

A parallel lane with the purified protein fraction was run with the above gel, was cut out before staining, and transferred onto a nitrocellulose paper at 80 V for 3 h. The blot was immunostained with anti-embryonic chicken brain GalT-4 or anti-bovine GT antibody using horseradish peroxidase conjugated second antibody. In brief, the nitrocellulose paper was first treated with 1% milk in phosphate-buffered saline (PBS) for 1 h at 37 °C, to minimize non-specific binding, followed by anti-GalT antibody at a specific dilution for the same period of time. The excess antibody was then washed off using 0.1% Tween 20 containing PBS (5 × 5 min). This blot was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h at 37 °C. After washing off excess antibody with Tween-20 containing PBS, the antibody-conjugated purified protein band was detected using 4-chloro-1-naphthol.

ENZYME ASSAY

The complete incubation mixtures contained the following components (in μmol) in a final volume of 0.04 ml: acceptor glycolipid LcOse3Cer, 0.01; detergent CF-54, 5–20 μg; HEPES buffer, 10, pH 7.2; $MnCl_2$, 0.125; UDP-[^{3}H]Gal, 0.012 (4–5 × 10^6 cpm per μmol), and enzyme protein 10–50 μg. After incubation for 1–2 h at 37 °C, the total incubation mixture was spotted on SG-81 or Whatman 3MM paper and chromatographed in 1% sodium tetraborate following the published method [6]. Product formation was quantitated from appropriate areas of each chromatogram with a Beckman Scintillation Counter LS-3133T.

CHARACTERIZATION OF RADIOACTIVE PRODUCTS

Large batches of enzymatic products were obtained from 20-fold increased incubations, and purified using Sep-Pak and Biosil columns [6]. The purity of the radioactive products was checked by TLC with authentic glycolipids in chloroform:methanol:water (50:40:10). The anomeric linkages in the products were determined by the cleavage pattern with various galactosidases [36]. Radioactive products were characterized by immunostaining using monoclonal antibodies against Type 1 and Type 2 GSLs following a modified method [46].

Results

Requirements for GalT-4

The membrane-bound mouse lymphoma GalT-4 exhibited an absolute requirement for detergent, and both neutral (Triton CF-54, Triton X-100) and cationic (taurodeoxycholate) detergents seemed to be equally effective. The membrane-bound enzyme solubilized using neutral detergent Triton CF-54 did not exhibit an absolute requirement for externally added detergent for optimal activity. Like other galactosyltransferases, Mn^{2+} is essential for mouse lymphoma GalT-4 but could be partially replaced by Mg^{2+}, Ca^{2+}, or Fe^{3+}. The reaction is completely inhibited by addition of EDTA. The enzyme activity is proportional to time up to 3 h at a protein concentration between 0.1 and 0.2 mg for membrane-bound enzyme. However, for the highly purified fraction, a fg amount of protein is used.

Purification

The purification procedure for the GalT-4 from mouse lymphoma is shown in Table 1. The detergent-soluble supernatant is passed through two successive alpha-lactalbumin-sepharose 4B affinity columns following the procedure described in the Methods section. The GalT-4 activity binds to the column and is eluted by washing with buffer in absence of both *N*-acetylglucosamine and Mn^{2+}. The enzymatically active eluent from the second column is further purified (107 500-fold purification) by affinity

Table 1. Purification of mouse lymphoma GalT-4.

Steps	*Total volume (ml)*	*Total protein (mg)*	*Total units (nmol min^{-1})*	*Specific Activity (U mg^{-1})*	*Fold*	*Yield (%)*
Homogenate	510	6400	80	0.0125	1.0	100
Residue	402	3911	71.6	0.018	1.4	89.5
Det. Sol. Sup.	600	1070	76.6	0.07	5.6	95.7
Lact-El. 1	107	1.2	20.8	17.3	1386	26.0
Lact-El. 2	20	0.04	19.1	479.0	38320	23.8
UDP-El.	0.6	0.0031	4.2	1344.0	107526	5.2

chromatography through a UDP-sepharose column. The purified GalT-4 is very unstable and loses its enzymatic activity within 48 h. However, the enzymatic activity could be partially stabilized in the presence of *N*-acetylglucosamine and Mn^{2+}, at least for 2 weeks at 4 °C.

In an attempt to stabilize the most pure form of enzyme for different kinetic and functional studies, we have tried to create an artificial membrane environment by introducing phospholipid-liposomes during enzyme assay [39]. We observed some stabilizing effects with both phosphatidylethanolamine and phosphatidylcholine at a moderate concentration (up to 1 mg ml^{-1}) with purified GalT-4 (Fig. 1) while phosphatidylserine was inhibitory at that concentration. A similar phospholipid effect has been reported for glycoprotein biosynthesis and bovine milk GT [47, 48].

The highly purified fraction from P-1798 was subjected to 8% SDS-PAGE in the presence of β-mercaptoethanol. Two closely migrating protein bands at 57 kD and 63 kD were visualized after silver staining (Fig. 2a, Lane 2). Both silver-stained bands gave positive immunostaining when tested with polyclonal antibody against purified bovine milk β4GT (Fig. 2a, Lane 3). In order to investigate whether these two protein bands are the result of proteolysis, protein fingerprinting analyses were performed using *Staphylococcus aureus* V8 protease as described in the text. As seen in Fig. 2b, three peptide bands with identical mobilities are observed after limited proteolysis indicating amino acid sequence identity between the resulting peptides. Similar results were obtained with HeLa cells GT species as three peptide bands were observed after V8 protease digestion of the three closely migrated immunoprecipitated protein bands [49].

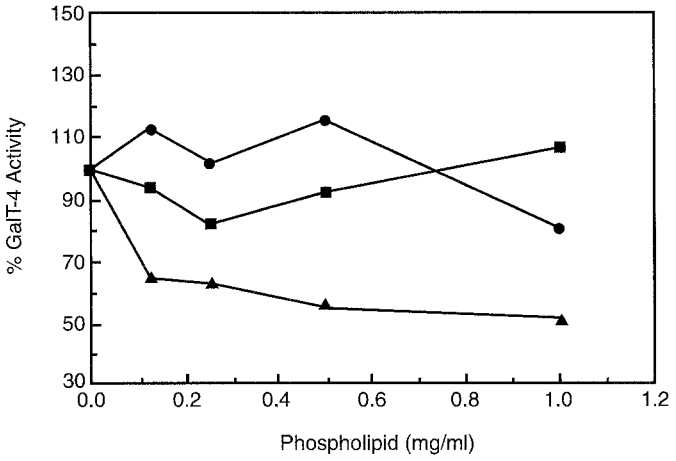
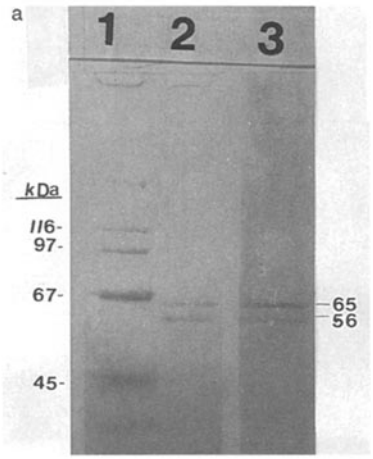


Figure 1. Effect of phospholipids on purified GalT-4. The stability of purified GalT-4 from P-1798 is shown in Fig. 2. The enzyme was preincubated at 37 °C with different phospholipids as indicated in the figures, according to a recently published method [37] followed by incubation under regular incubation conditions for 2 h. The results are expressed as a percentage of original 100% activity (30 nmol per mg protein per 2 h). The phospholipids are represented in the figure as follows: phosphatidyl-ethanolamine (PE, ■); phosphatidylcholine (PC, ●) and phosphatidyl-serine (PS, ▲).

Substrate specificity

The substrate specificity studies with various potential glycolipids indicate that LcOse3Cer is the most preferred acceptor for P-1798 GalT-4 as shown in Table 2. It is important to note complete absence of the galactosyltransferase, GalT-3 (UDP-Gal: GM2 β1-3galactosyltransferase), reported from embryonic chicken brain [50, 51], which catalyzes the biosynthesis *in vitro* of ganglioside GM1 in the purified enzyme fraction from mouse tumour. In addition to LcOse3Cer, the only other comparatively effective substrate seems to be iLcOse5Cer (GlcNAcβ1-3nLcOse4-Cer), the precursor for i-antigen and also for polylactosamine structures, with about 28% activity; all other acceptors are completely inactive. The K_m values for the substrates LcOse3Cer and iLcOse5Cer are 180 μM and 100 μM, respectively, while that for the donor nucleotide sugar UDP-galactose is 250 μM (data not shown). Competition studies were performed using two active substrates with the purified enzyme from P-1798 GalT-4. As shown in Table 3, the experimental value corresponds closely to the theoretical value calculated for two enzymes [52], when mixed substrates (LcOse3Cer and iLcOse5Cer) are used. Whether these two reactions are indeed catalysed by two separate proteins is under investigation, since the most purified protein fraction contains two closely migrating bands.

As shown in Table 2 asialo-agalacto-α1-acid glycoprotein (SA$^-$Gal$^-\alpha_1$AGP), *N*-acetylglucosamine (GlcNAc),



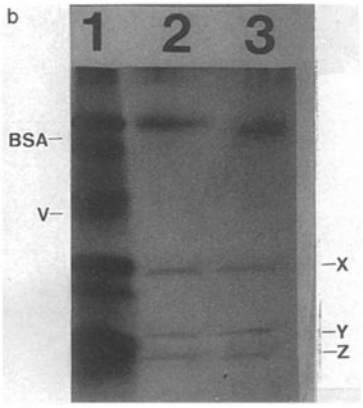


Figure 2. SDS-PAGE/Western Blot-Immunoblot of Purified GalT-4. (a) Silver staining profile of the purified GalT-4 from P-1798 is shown in Fig. 2a after 8% SDS-PAGE. The purified protein was electrophoresed in Lane 2, and two closely migrating bands at 56 and 63 kDa are observed as shown in the Figure. The proteins were electrotransferred to nitrocellulose paper from a parallel gel, run under the same condition followed by immunoblotting with anti-bovine milk GalT antibody. Both silver-stained bands of P-1798 GalT-4 resulted in a positive immunostained bands (Lane 3). A mixture of standard proteins was run in Lane 1 (in kDa: β-galactosidase, 116; phosphorylase b, 97; bovine albumin, 66; ovalbumin, 45; carbonic anhydrase, 29). (b) Two closely migrating silver-stained bands were subjected to limited proteolysis with V8 protease and electrophoresed in 14% polyacrylamide gels. As seen in Fig. 2b, both 63 kDa (Lane 2) and 56 kDa (Lane 3) bands are cleaved with the production of three peptides of similar mobilities (X, Y, Z). Lane 1 has a mixture of low molecular weight standards (Sigma). Migration of V8 protease is indicated by V.

Table 2. Substrate specificity of GalT-4. The incubation mixture contained the following components (in μmol) in a final volume of 0.04 ml: potential acceptor glycolipids, glycoprotein or free GlcNAc and p-nitrophenyl-GlcNAc as indicated; Triton CF-54, 5–10 μg; HEPES buffer, 10, pH 7.2; $MnCl_2$, 0.25; UDP-Gal, 0.012 (1.2×10^6 cpm μmol^{-1}); 10–20 ng protein purified fraction from P-1798. The mixtures were assayed by the chromatographic method after 2 h incubation. The endogenous values were corrected from values for each substrate.

Substrate	*Abbreviations*	*Concentrated (mM)*	*[^{3}H]Galactose incorporated (μmol per mg per 2 h)*
(GlcNAcβ1-3Lc2)	Lc3	0.25	23.5
(GlcNAcβ1-3nLc4)	iLc5	0.25	8.1
(GalNAcβ1-4Lc2)	Gg3	0.25	<endogenous
(GalNAcβ1-4(α2-3NeuAc)Lc2)	GM2	0.25	<endogenous
$SA^-Gal^-\alpha_1$AGP		0.62 mg ml^{-1}	10.2
GlcNAc		4 mM	40.1
pNP-GlcNAc		1.25 mM	42.7

Table 3. Substrate competition studies of mouse lymphoma GalT-4 with potential glycolipid acceptors. Substrate competition experiments were performed with the two most preferred glycolipid substrates in the presence of purified mouse lymphoma enzyme. Experimental conditions were the same as described for Table 2 except mixed substrates were used for competition experiment. Chromatographic assay method was used for quantitation of the radioactive products.

				Theoretical value for	
Enz. Fr.	*Substrate*	*Concentration (mM)*	V_{exp}	*One enzyme (nmol Gal incorporated per ml per 2 h)*	*Two enzyme*
				$V_t = \frac{V_a(a/K_a) + Vb(b/K_b)}{1 + a/Ka + b/Kb}$	$V_t = Va + Vb$
Purified Enzyme	Lc3	0.125	18.5		
Purified Enzyme	iLc5	0.125	5.6		
Purified Enzyme	Lc3 + iLc5 (each)	0.125	25.8	6.7	24.1

and p-nitrophenyl-*N*-acetylglucosamine (pNP-GlcNAc) all exhibited good acceptor profiles for the mouse lymphoma GalT-4. However, the K_m for *N*-acetylglucosamine is much higher (3.5 mM) than for all other substrates (K_m for $SA^{-}Gal^{-}\alpha_1AGP$, 0.4 mg ml^{-1}; for pNP-GlcNAc, 0.4 mM). The substrate competition studies with the glycoprotein substrate and with free saccharide in the presence of Lc3 indicated that all these reactions might be catalysed by the same protein since the experimental values are closer to the theoretical values for one enzyme as shown in Table 4 [52].

The glycolipid:glycosyltransferases can be classified on the basis of their substrate recognition site requirement as reported recently [53, 54]. In order to establish the minimum structural requirement for the substrate, we have used lyso (deactylated-oligosaccharide-sphingosine) as well as reacetylated lyso (oligosaccharide-acetylsphingosine) acceptor glycolipd, LcOse3Cer [37, 38], with the purified enzyme. The reacetylated lyso substrate, where the fatty acyl group of ceramide is replaced by acetyl-moiety, behaves as a moderately good acceptor with only 1.5- to three-fold higher K_m (250 μM: data not shown). This probably suggests that the enzyme does not have a specific requirement for fatty acid side chain while the presence of *N*-acetylglucosamine moiety in the acceptor glycolipid is absolutely necessary, since deacetylated lyso substrate is completely inactive as an acceptor.

Analysis of the radioactive products

Autoradiography of the radioactive products, prepared from 20-fold increased incubation mixtures using the most purified enzyme, indicated the presence of nLcOse4Cer since the radioactive band cochromatographed with authentic glycolipid as shown in Fig. 3. Radioactive products have been characterized further (data not shown) by both immunostain on thin layer chromatography [46]

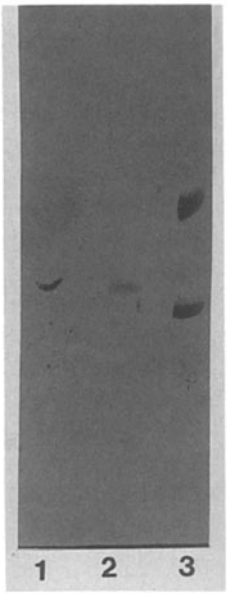


Figure 3. Autoradiography of [^{3}H] labeled glycolipid product from the GalT-4 catalysed reaction. Lane 1, Standard nLcOse4Cer; Lane 2 [^{3}H] labeled glycolipid product (3000 cpm) obtained from α-lactalbumin column purified GalT-4; Lane 3, mixture of standard LcOse3Cer and nLcOse5Cer.

and ELISA using the monoclonal antibody IB2 specific for nLcOse4Cer following the previously published protocol from our laboratory [55]. The purified radioactive product has also been subjected to acetylation in pyridine-acetic anhydride at 60 °C overnight [56] to determine whether the radioactive product is a mixture of Type 1 (Galβ1-3GlcNAc-R) and Type 2 (Galβ1-4GlcNAc-R) isomers. Following acetylation, the products are separated with chloroform after neutralizing to pH 7.0. Acetylated radioactive product formed by purified P-1798 GalT-4

Table 4. Substrate competition studies of mouse lymphoma GalT-4 with potential glycoprotein and oligosaccharide acceptors. Substrate competition experiments were conducted with the potential glycoprotein and free saccharide substrates with the purified lymphoma enzyme. Experimental conditions were the same as indicated in Table 2 except indicated amounts of substrate concentrations were used. The instability of the purified enzyme could contribute to the lower experimental values compared to Table 3.

			Theoretical value for	
Substrate	*Concentration (mM)*	V_{observ}	*One Enzyme (nmol Gal incorporated per ml per 2 h)*	*Two Enzymes*
			$V_t = \frac{V_a(a/K_a) + Vb(b/K_b)}{1 + a/Ka + b/Kb}$	$V_t = Va + Vb$
Lc3	0.2	4.2		
GlcNAc	3.5	11.24		
$SA^{-}Gal^{-}\alpha_1AGP$	0.5 mg ml^{-1}	2.8		
Lc3 +	0.2			
$SA^{-}Gal^{-}\alpha_1AGP$	0.5 mg ml^{-1}	2.3	2.4	7.0
Lc3 + GlcNAc	0.2 + 3.5	6.2	5.38	15.44

comigrated with authentic acetylated nLcOse4Cer as seen after thin layer chromatography in chloroform:methanol (95:5) confirming the formation of Galβ1-4 linkage in the products (data not shown).

Discussion

The mouse lymphoreticular system has been used extensively for the study of melanoma [57]. Mouse haematopoeitic cells of both B- and T-origin arrested at different stages of development have been induced either chemically or virally to produce tumours [58] and thus provide an excellent model system to perform a systematic study of tumorigenic glycolipid biosynthesis. Previously, a brief study had been undertaken to evaluate and assess the activities of several glycosyltransferases in mouse lymphoma of both B- and T-origin [13, 59]. The results presented here describe the purification of galactosyltransferase GalT-4, which catalyses the biosynthesis *in vitro* of nLcOse4Cer, from a mouse tumour, a mineral oil induced mouse T-lymphoma. We have been able to purify the mouse lymphoma GalT-4 107 500-fold using affinity columns. The most purified protein fraction resulted in two closely migrating protein bands when subjected to SDS-PAGE, and both the bands showed positive immunostaining with anti-bovine β4GT (EC.2.4.1.22). We have used an antibody raised against purified GalT-4 from embryonic chicken brain [14] which also showed similar results when tested with the purified mouse enzyme. These results suggest at least partial structural similarity of this particular galactosyltransferase among different species. Protein fingerprinting analysis of these two closely migrating bands (56 kDa and 63 kDa) from mouse lymphoma indicated that both the bands have homologous amino acid sequences since the resulting peptides from each showed similar electrophoretic mobilities. These results are in accordance with similar observations previously reported with HeLa cell GT species [49] indicating that mouse lymphoma GalT-4 is similar to UDP-Gal:GlcNAc-R β4GT (EC.2.4.1.38). The stability of the enzyme, however, gradually decreases with each step of purification and the most purified protein fraction (107 500-fold) becomes extremely unstable. The addition of phospholipids partially stabilizes the activity of the purified protein. Similar results were previously reported with purified rat mammary gland galactosyltransferase [47, 60]. The GalT-3 galactosyltransferase from embryonic chicken brain, which catalyses biosynthesis *in vitro* of GM1 ganglioside, has also been shown to be stabilized by addition of phospholipid [14, 39, 51]. In addition the fatty acid chains of the phospholipids also play a significant role in the modulation of enzymatic activity [39].

The requirements for the mouse lymphoma GalT-4 resemble those of all other GalT-4, in that the presence of Mn^{2+} is essential for enzyme activity [5, 6]. The Mn^{2+} requirement could be partially replaced by Mg^{2+}, Ca^{2+}, and Fe^{3+}, and EDTA inhibits the activity.

The substrate specificity studies with the purified mouse lymphoma GalT-4 indicated that the enzyme is equally active with GlcNAc-terminal-containing glycolipid and glycoprotein as well as free saccharide (Table 2). Substrate competition studies with glycolipid (Lc3) in combination with either glycoprotein ($SA^{-}Gal^{-}\alpha_1$AGP) or free saccharide (GLcNAc) indicated that the same protein is probably catalysing all the reactions as evidenced by the observed V_{max} which is close to the theoretical value calculated for one enzyme (Table 4). This particular kind of substrate specificity of β4GT (EC.2.4.1.38) has not been shown before. However, in addition to LcOse3Cer, the only other glycolipid substrate which showed acceptor activity was iLcOse5Cer, the precursor for polylactosamine core antigen, with about 28% activity compared to that with LcOse3Cer (Table 2). It is expected that these two reactions might be catalysed by the same protein. However, the substrate competition studies, using both these substrates in combination, indicated that two separate gene products might be responsible for catalysing these two reactions (Table 3).

These intriguing results correlate with observations made for the preceding enzyme in the biosynthetic route. The *N*-acetylglucosaminyltransferases GlcNAcT-1 and GlcNAcT-2 (Fig. 4), the enzymes which catalyse biosynthesis *in vitro* of LcOse3Cer and iLcOse5Cer, respectively, in human colon carcinoma cells Colo-205,

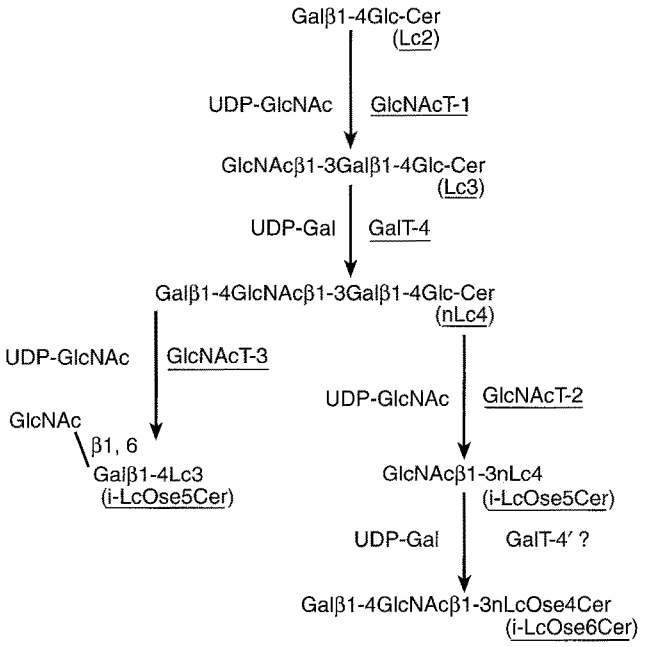


Figure 4. Proposed pathway for the stepwise biosynthesis *in vitro* of i/I-antigens in mouse T-lymphoma P-1798

were reported to be catalysed by two different gene products [61]. Previously, it has been shown, using mouse T-lymphoma P-1798 Golgi-membrane as enzyme source, that GlcNAcT-3, which catalyses the formation of GlcNAcβ1-6nLcOse4Cer, the branched li-core precursor, is a separate gene product from GlcNAcT-2 [62]. Similar observations were also made with human serum GlcNAcTs [63] and also with GlcNAcTs from Novikoff ascites tumour cells [64]. It is tempting to speculate that the glycosyltransferases which catalyse the biosynthesis of the type 2 antigen core nLcOse4Cer, in a stepwise fashion (GlcNAcT-1, GalT-4; Fig. 4) starting from lactosylceramide LcOse2Cer, might be a set of separate gene products from the glycosyltransferases which are involved in the modification of the core structure by extension or branching with the formation of longer or branched structures (GlcNAcT-2, GlcNAcT-3, GalT-4'?; Fig. 4).

The galactosyltransferases comprise a group of enzymes which catalyse the transfer of galactose from UDP-galactose to specific acceptors with the formation of products containing specific linkages. It is well established that each specific linkage is catalysed by a specific gene product. The GalT-3, which catalyses the formation of GM1 ganglioside from GM2 [50, 51], seems to be totally absent from the purified enzyme GalT-4 fraction as shown in Table 2. The galactosyltransferase GalT-2, which catalyses the formation of lactosylceramide, is a distinct protein and separate from GalT-4. This enzyme was originally reported from embryonic chicken brains [65], and recently the same protein has been purified to homogeneity from human kidney proximal tubular cells [66].

The appearance of fucosylated- and sialylated-polylactosamine glycolipid antigens during oncodevelopmental processes is well documented in the literature [1–3]. The importance of core glycolipid neolactotetraosylceramide is increasing; and, in turn, the role of GalT-4, the enzyme catalysing the formation of the core antigen, is also under significant attention. Elevated levels of GalT-4 have been reported recently in the serum of a cancer patient [67] and rat prostate cancer cells [16]. Also reported recently is the correlation of the expression of GalT-4 with the development of the nervous system [68].

The general trend of thought until now has been that the same GalT-4 is responsible for the synthesis of the polylactosamine repeat unit although no experimental data have been published as yet. The result presented here clearly suggests that the synthesis of nLcOse4Cer and nLcOse6Cer may be catalysed by two homolgous proteins. The two closely migrating bands in the purified enzyme fraction seem to have a significant similarity in amino acid sequence homology according to the protein fingerprinting analyses (Fig. 2b). It is also possible that post-translational modification of a single protein, like glycosylation or phosphorylation, might be necessary for its dual specificity toward the substrate.

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