Rat liver Golgi galactosyltransferases

Distinct enzymes for glycolipid and glycoprotein acceptor substrates

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1. Two enzymes that catalyse the transfer of galactose from UDP-galactose to G_{M2} ganglioside were partially purified from rat liver Golgi membranes. 2. These preparations, designated enzyme I (basic) and enzyme II (acidic), utilized as acceptors G_{M2} ganglioside and asialo G_{M2} ganglioside as well as ovalbumin, desialodegalactofetuin, desialodegalacto-orosomucoid, desialo bovine submaxillary mucin and G_{M2} oligosaccharide. Enzyme II catalysed disaccharide synthesis in the presence of the monosaccharide acceptors *N*-acetylglucosamine and *N*-acetylgalactosamine. 3. The affinity adsorbent α -lactalbumin-agarose, which did not retard G_{M2} ganglioside galactosyltransferase, was used to remove most or all of galactosyltransferase activity towards glycoprotein and monosaccharide acceptors from the extracted Golgi preparation. 4. After treatment of the extracted Golgi preparation with α -lactalbumin-agarose, enzyme I and enzyme II G_{M2} ganglioside galactosyltransferase activities, prepared by using DEAE-Sepharose chromatography, were distinguishable from transferase activity towards G_{M2} oligosaccharide and glycoproteins by the criterion of thermolability. 5. This residual galactosyltransferase activity towards glycoprotein substrates was also shown to be distinct from G_{M2} ganglioside galactosyltransferase in both enzyme preparations I and II by the absence of competition between the two acceptor substrates. 6. The two types of transferase activities could be further distinguished by their response to the presence of the protein effector α -lactalbumin. G_{M2} ganglioside galactosyltransferase was stimulated in the presence of α -lactalbumin, whereas the transferase activity towards desialodegalactofetuin was inhibited in the presence of this protein. 7. The results of purification studies, comparison of thermolability properties and competition analysis suggested the presence of a minimum of five galactosyltransferase species in the Golgi extract. Five peaks of galactosyltransferase activity were resolved by isoelectric focusing. Two of these peaks (pI 8.6 and 6.3) catalysed transfer of galactose to G_{M2} ganglioside, and three peaks (pI 8.1, 6.8 and 6.3) catalysed transfer to glycoprotein acceptors.

Abbreviations used: G_{M2} ganglioside, GalNAc β 1 $\rightarrow$ 4Gal β 1 $\rightarrow$ (3 $\leftarrow$ 2 α NeuAc)Glc β 1 $\rightarrow$ 1ceramide; G_{M1} ganglioside, Gal β 1 $\rightarrow$ 3GalNAc β 1 $\rightarrow$ 4(3 $\leftarrow$ 2 α NeuAc)Glc β 1 $\rightarrow$ 1ceramide; asialo G_{M2} ganglioside, GalNAc β 1 $\rightarrow$ 4Gal β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1ceramide; G_{M2} oligosaccharide, GalNAc β 1 $\rightarrow$ 4Gal(3 $\leftarrow$ 2 α NeuAc)Glc; G_{D2} ganglioside, GalNAc β 1 $\rightarrow$ 4Gal(3 $\leftarrow$ 2 α NeuAc8 $\leftarrow$ 2 α NeuAc) β 1 $\rightarrow$ 4Glc β 1 $\rightarrow$ 1ceramide; DSDG fetuin, desialodegalactofetuin; DSDG orosomucoid, desialodegalacto-orosomucoid; DS bovine submaxillary

mucin, desialo bovine submaxillary mucin; GalNAc, *N*-acetylglactosamine; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; α Lac, α -lactalbumin.

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The solubilization and partial purification of two isoenzymes of G_{M2} ganglioside galactosyltransferase from rat liver Golgi membranes have recently been described (Kaplan & Hechtman, 1983). Characterization of enzyme I (basic) and enzyme II (acidic) indicated that the two enzymes had distinct pH optima and detergent and phospholipid requirements, but similar cation requirements, molecular masses and kinetic constants. Among sphingolipid substrates only G_{M2} and asialo G_{M2} gangliosides served as acceptors for galactose. In the present study we have investigated acceptor specificities of enzyme I and enzyme II by using protein-separation techniques, competition analysis and comparison of properties of enzyme activities that transfer galactose to different acceptor substrates.

With the exception of the inner core of asparagine-linked oligosaccharides of complex glycoproteins, the addition of monosaccharides to glycolipids, mucin-type glycoproteins and terminal sugar residues of complex glycoproteins occurs by the stepwise addition of sugar residues from nucleotide sugar donors catalysed by glycosyltransferases.

The galactosyl linkages formed in the three types of oligosaccharide are all in the β -configuration and are linked either to the 4-position of *N*-acetylglucosamine or to the 3-position of *N*-acetylgalactosamine. The oligosaccharide chains associated with proteins are classified either as mucins or as complex glycoproteins. In mucins the *N*-acetylhexosaminyl residue is linked directly to the oxygen atom of serine or threonine. In complex glycoproteins the inner *N*-acetylglucosaminyl residue is attached to the nitrogen atom of asparagine. The core structure of complex glycoproteins possesses a branched mannose trisaccharide, and the terminal carbohydrate sequence of *N*-acetylhexosamine, galactose and *N*-acetylneuraminic acid is added in the Golgi apparatus.

A characteristic feature of glycosyltransferase enzymes is their high order of specificity for donor and acceptor substrates. The substrate specificities of a variety of glycoprotein sialyl-, galactosaminyl-, glucosaminyl-, fucosyl- and galactosyl-transferases have been described in detail (Schachter & Roseman, 1980).

G_{M2} , G_{D2} and asialo G_{M2} gangliosides have been shown to be effective acceptors for galactose transfer catalysed by particulate fractions from embryonic chick brain (Basu *et al.*, 1965), rat brain (Cumar *et al.*, 1972; Dicesare & Dain, 1972) and rat liver Golgi membranes (Keenan *et al.*, 1974). Competition analysis has shown that the transfer of galactose to glycoprotein acceptor substrates catalysed by this crude preparation is due to enzymes distinct from those catalysing incorpora-

tion of galactose into glycolipid acceptors (Basu *et al.*, 1965).

Schachter *et al.* (1971) measured galactosyltransferase activity towards G_{M2} ganglioside in a particulate preparation obtained from pig submaxillary gland that was active towards DS mucin substrates possessing *N*-acetylgalactosaminyl termini. Activity towards the two types of acceptors could be distinguished by failure of the two substrates to compete.

These efforts indicate the limited extent to which the acceptor specificities of enzymes catalysing galactose transfer to glycolipid acceptors have been analysed. In the present investigation we have examined differences in properties between glycolipid galactosyltransferases and glycoprotein galactosyltransferases extracted from rat liver Golgi membranes.

Materials and methods

Materials

D-UDP-[U- ^{14} C]galactose (274 mCi/mmol) and [^{3} H]sucrose (1–5 mCi/mmol) were obtained from New England Nuclear Corp. (Montreal, Quebec, Canada). UDP-galactose, fetuin (type IV from foetal-calf-serum), mucin (type I from bovine submaxillary glands), orosomucoid (human, purified from Cohn fraction VI), α -lactalbumin (bovine milk), galactosyltransferase (lactose synthase, EC 2.4.1.22), *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, α -lactalbumin-agarose (1 mg of α -lactalbumin/ml of gel) and *N*-acetylneuraminyl-lactose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE-Sephadex 6B and Sephadex G-10 were purchased from Pharmacia (Uppsala, Sweden).

Bio-Gel P-2 was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Hen's-egg ovalbumin was generously given by Dr. S. Mookerjee (Memorial University, St. John's, Newfoundland, Canada). Samples of sheep submaxillary mucin and orosomucoid from which sialic acid and galactose had been removed were kindly furnished by Dr. H. Schachter (Hospital for Sick Children, Toronto, Ontario, Canada).

A sialohexasaccharide isolated from the urine of a patient with a confirmed case of sialidosis was prepared by Dr. N. M. K. Ng Ying Kin (Montreal Neurological Hospital, Montreal, Quebec, Canada).

Preparation of substrates

G_{M2} ganglioside and asialo G_{M2} ganglioside were prepared as described previously (Folch *et al.*, 1957; Wolfe, 1972; Suzuki & Suzuki, 1972). G_{M2} ganglioside was labelled in the *N*-acetylgalactosaminyl moiety by using the procedure developed

by Suzuki & Suzuki (1972) as modified by O'Brien *et al.* (1977) and Novak *et al.* (1979). The specific radioactivity was 1.5mCi/mmol. An oligosaccharide was prepared from ^3H -labelled $\text{G}_{\text{M}2}$ ganglioside by ozonolysis (Esselman *et al.*, 1972). The ozonolysed products were resolved by gel filtration on Bio-Gel P-2. The presence of the sialotetrasaccharide was confirmed by t.l.c. in butanol/acetic acid/water (4:1:1, by vol.). A single resorcinol-positive spot was observed with mobility intermediate between that of *N*-acetylneuraminyl-lactose and the sialohexasaccharide.

Preparation of glycoprotein acceptors

Sialic acid was removed from fetuin, orosomucoid and bovine submaxillary mucin by mild acid hydrolysis. Removal of galactosyl termini from DS fetuin and DS orosomucoid was achieved by periodate oxidation followed by reduction with NaBH_4 and mild acid hydrolysis, in accordance with the procedure of Spiro (1964).

Enzyme assays

Measurement of enzymic synthesis of $\text{G}_{\text{M}1}$ ganglioside was performed as previously described (Kaplan & Hechtman, 1983). Reaction conditions for the measurement of galactose incorporation into glycoprotein acceptors were identical except that suitably prepared acceptors (125–250 μg) were substituted for $\text{G}_{\text{M}2}$ ganglioside.

Labelled reaction products were separated and quantified as described in a previous paper (Kaplan & Hechtman, 1983).

Zero-time incorporation was determined by immediate addition of precipitant to reaction mixtures. The total radioactivity added to reaction mixtures was measured by direct application of diluted UDP-[U- ^{14}C]galactose to a Millipore filter containing the precipitated reaction components.

Oligosaccharide biosynthesis was assayed by separation of doubly labelled [^3H , ^{14}C]oligosaccharide product from ^3H -labelled $\text{G}_{\text{M}2}$ oligosaccharide and UDP-[U- ^{14}C]galactose by using high-voltage paper electrophoresis. After incubation, reaction mixtures, which contained 26nmol of oligosaccharide, were electrophoresed in 2% (w/v) sodium tetraborate, pH 9.0, for 2 h at 1500 V. Electrophoretograms were cut into 6 mm strips and radioactivity was determined in a liquid-scintillation spectrometer with the use of double-label settings. ^{14}C spill into the ^3H channel was in the range of 10–17%. There was no measurable spill of ^3H into the ^{14}C channel.

For measurement of disaccharide synthesis incubation mixtures containing 1 mM-*N*-acetylglucosamine or *N*-acetylgalactosamine were mixed with 10 mg of wet charcoal for adsorption of UDP-

[U- ^{14}C]galactose, and the donor-free reaction mixture was chromatographed on a Sephadex G-10 column (23 cm $\times$ 1 cm). The gel-filtration column was calibrated with Blue Dextran, uridine, [^3H]sucrose and UDP-[U- ^{14}C]galactose. Disaccharide products were measured by determination of radioactivity co-eluted with sucrose.

Protein determinations

Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard. Where necessary, a modified Lowry procedure was adopted to adjust for high Triton X-100 concentrations (Wang & Smith, 1965).

Preparation of enzymes

Tissue sources. Three-week-old rats were obtained from Canadian Breeding Farms and maintained on water only before they were killed. Enzymes I and II were prepared from rat liver Golgi membranes by anion-exchange chromatography as described previously (Kaplan & Hechtman, 1983). For the preparation of enzyme $\text{I}_{\alpha\text{Lac}}$ and enzyme $\text{II}_{\alpha\text{Lac}}$ the solubilized Golgi preparation (S_{105}) was initially chromatographed on α -lactalbumin-agarose. The fractions containing unbound $\text{G}_{\text{M}2}$ ganglioside galactosyltransferase activity were pooled ($\text{S}_{\alpha\text{Lac}}$) and chromatographed on DEAE-Sepharose 6B by using a procedure identical with that described in Kaplan & Hechtman (1983).

α -Lactalbumin-agarose chromatography. Solid KCl and *N*-acetylglucosamine were added to 5 ml of an S_{105} preparation to final concentrations of 20 mM and 3 mM respectively. The sample was applied to a 2 ml column (2.5 cm $\times$ 1 cm) of α -lactalbumin-agarose equilibrated in 10 mM-Tris/HCl buffer, pH 7.6, containing 10 mM- MgCl_2 , 20 mM-KCl, 2 mM-2-mercaptoethanol, 3 mM-*N*-acetylglucosamine and 1% (v/v) Triton X-100, and 1 ml fractions were collected manually at a flow rate of 1 ml/min. Unadsorbed protein was eluted with 15 ml of equilibration buffer. Bound galactosyltransferase was eluted with the same buffer devoid of *N*-acetylglucosamine. The column was calibrated with a commercial preparation of bovine milk galactosyltransferase.

Isoelectric focusing. Isoelectric focusing was performed in a Pharmacia flat-bed apparatus. To 100 ml of Sephadex G-75 swollen in 1% (v/v) Triton X-100/50% (v/v) glycerol was added 3.3 ml of ampholytes (Pharmalyte 3.0–10.0), and the gel was poured and prefocused for 45 min at 20 mA at 750 V. A 1 ml sample of an S_{105} preparation was applied and the focusing was continued for 16 h. Equilibrium was reached when two spots of myoglobin that had been applied at opposite ends of the gel had coalesced. Gel fractions were re-

suspended in 500 μ l of equilibration buffer and centrifuged at 300g for 10 min. The supernatants were assayed immediately for galactosyltransferase activity.

Results

Presence of multiple galactosyltransferases in rat liver Golgi membranes

Galactosyltransferase activities towards a variety of acceptor substrates were assessed in Triton X-100-solubilized Golgi extract (S_{105}) and in anion-exchange-chromatographic-column fractions containing enzymes I and II. The results are summarized in Table 1. Glycoproteins with either *N*-acetylglucosaminyl termini (ovalbumin, DSDG orosomucoid), *N*-acetylgalactosaminyl termini, (DS bovine submaxillary mucin) or both (DSDG fetuin) served as excellent acceptors of galactose in the presence of all three enzyme preparations. With all four glycoprotein acceptors the specific activity of galactose transfer was greater than that measured in the presence of G_{M2} ganglioside. The concentrations of exposed *N*-acetylhexosaminyl residues in reaction mixtures containing the different acceptors were not limiting factors in the galactosyltransferase reactions. From the *N*-acetylhexosamine content in terminal positions of the acceptors listed in Table 1, we have calculated that the percentage of such residues galactosylated in these reaction mixtures ranged from a minimum of 0.07% (for DG bovine submaxillary mucin) to a maximum of 7.4% (for G_{M2} oligosaccharide). Native fetuin, DS fetuin, mucin and orosomucoid were not acceptors of galactose.

Incorporation of galactose into labelled disaccharide was measured in the presence of *N*-acetyl-

glucosamine or *N*-acetylgalactosamine with DEAE-Sepharose-column fractions containing enzyme I or enzyme II as the enzyme source. Both monosaccharides were highly effective acceptors of galactose when incubated with fractions containing enzyme II. Disaccharide biosynthesis was recorded as radioactivity co-eluted with [^{3} H]-sucrose in reaction mixtures containing monosaccharide acceptors (Figs. 1a and 1b) minus radioactivity co-eluted with sucrose in reaction mixtures containing no acceptor (Fig. 1c). No attempt was made to characterize the position of the linkages formed or to determine the nature of the product(s) formed in the absence of exogenous acceptors. Incorporation of galactose in the presence of *N*-acetylgalactosamine was about twice that of incorporation in the presence of *N*-acetylglucosamine.

Removal of a lactose synthase-like enzyme by α -lactalbumin-agarose chromatography.

The multiple acceptor specificities present in the partially purified enzymes I and II indicated that both of these preparations contained a number of unresolved galactosyltransferases. One species of galactosyltransferase that has been extensively purified from rat liver microsomal fraction (Fraser & Mookerjee, 1977) is an enzyme that is specific for *N*-acetylglucosamine or for glycoprotein acceptors containing terminal β -(1 $\rightarrow$ 1)-*N*-acetylglucosaminyl linkages. The specificity of this enzyme is modified by the protein effector α -lactalbumin, and this interaction has become the basis of purification of this lactose synthase enzyme by affinity chromatography on α -lactalbumin-agarose.

The solubilized Golgi extract (S_{105}) was chromatographed on α -lactalbumin-agarose under

Table 1. *Acceptor specificity of rat liver Golgi galactosyltransferases*

Assay mixture for the measurement of galactosyltransferase activity contained 10 nmol of UDP-[U- ^{14} C]galactose (40 000 c.p.m.), 15 μ mol of Tris/succinate buffer, pH 6.0, 2.5 μ mol of $MnCl_2$, 0.5 mg of Triton X-100, 25 mg of glycerol and acceptor substrate in a final volume of 100 μ l. G_{M2} ganglioside and asialo G_{M2} ganglioside were added to a final concentration of 0.5 mM and *N*-acetylglucosamine and *N*-acetylgalactosamine to 1 mM. For the measurement of glycoprotein galactosyltransferase activity 250 μ g of glycoprotein was added to reaction mixtures, except in the case of DSDG fetuin, where 125 μ g was assayed. Incubations were at 30°C for 2 h. Reaction products were isolated as described in the Materials and methods section. Abbreviations: N.D., not detected; —, not determined.

Substrate	Activity (nmol/2h per mg)		
	S_{105} preparation	Enzyme I	Enzyme II
G_{M2} ganglioside	4.3	9.4	11.2
Asialo G_{M2} ganglioside	4.7	9.3	10.8
DSDG fetuin	22.9	28.5	49.6
Ovalbumin	8.9	12.6	19.2
DSDG orosomucoid	11.9	22.0	17.1
DS bovine submaxillary mucin	21.2	68.1	13.6
<i>N</i> -Acetylglucosamine	—	N.D.	88.7
<i>N</i> -Acetylgalactosamine	—	N.D.	184.5

Fig. 1. Incorporation of galactose into labelled disaccharide in the presence of enzyme II and (a) *N*-acetylgalactosamine, (b) *N*-acetylglucosamine and (c) no acceptor substrate. Incubation conditions were as described in the Materials and methods section and in the legend to Table 1. After incubation, reaction mixtures were treated with charcoal as described in the Materials and methods section and applied to a column (23 cm $\times$ 1 cm) of Sephadex G-10. The column was eluted with distilled water. Forty 0.5 ml fractions were collected. The bracketed region shows the elution position of [^3H]sucrose.

conditions (Andrews, 1970) that retarded the bovine milk galactosyltransferase (Sigma). This enzyme is completely inactive towards $\text{G}_{\text{M}2}$ ganglioside. Over 80% of the $\text{G}_{\text{M}2}$ ganglioside galactosyltransferase activity recovered from the

column after application of the S_{105} preparation was eluted before the application of desorbing buffer (Fig. 2). A small portion of $\text{G}_{\text{M}2}$ ganglioside galactosyltransferase activity (10–20%) was retarded by the column and was eluted in the same position as the bovine milk galactosyltransferase. Two major peaks of galactosyltransferase with activity towards DSDG fetuin were clearly resolved by chromatography on α -lactalbumin-agarose (Fig. 2). The first peak, which represented 48% of recovered activity, was eluted together with $\text{G}_{\text{M}2}$ ganglioside galactosyltransferase. The second peak, representing 52% of the recovered activity, was adsorbed on the column and was eluted in a position identical with that of the bovine milk galactosyltransferase. The resolution of the glycoprotein-acceptor-specific galactosyltransferase activity of the Triton X-100-extracted Golgi membranes into two fractions on the α -lactalbumin-agarose column could not be an artifact due to column overloading. The application of 1000-fold greater amount of enzyme units of (Sigma) bovine milk lactose synthetase resulted in complete retardation of all enzyme in the presence of an *N*-acetylglucosamine-containing buffer.

Fractions that did not adsorb on the α -lactalbumin-agarose column and that had high $\text{G}_{\text{M}2}$ ganglioside galactosyltransferase activity were pooled ($\text{S}_{\alpha\text{Lac}}$) and chromatographed on DEAE-Sephacrose 6B (Fig. 3). The $\text{G}_{\text{M}2}$ ganglioside galactosyltransferase was recovered in two peaks, which chromatographed in a pattern identical with that observed when S_{105} preparation was chromatographed directly on DEAE-Sephacrose (Fig. 5 of Kaplan & Hechtman, 1983). Moreover, activity towards the glycoprotein DSDG fetuin was resolved into two peaks that were coincidental with the peaks of $\text{G}_{\text{M}2}$ ganglioside galactosyltransferase activity. These two peaks, prepared by a combination of α -lactalbumin-agarose chromatography and DEAE-Sephacrose chromatography, were designated enzyme $\text{I}_{\alpha\text{Lac}}$ and enzyme $\text{II}_{\alpha\text{Lac}}$.

Table 2 summarizes the rates of incorporation of galactose into substrates catalysed by $\text{S}_{\alpha\text{Lac}}$ and by DEAE-Sephacrose preparations containing enzyme $\text{I}_{\alpha\text{Lac}}$ and enzyme $\text{II}_{\alpha\text{Lac}}$ activities. Incorporation of galactose into ^3H -labelled $\text{G}_{\text{M}2}$ oligosaccharide catalysed by DEAE-Sephacrose fractions containing enzyme $\text{I}_{\alpha\text{Lac}}$ and enzyme $\text{II}_{\alpha\text{Lac}}$ was measured by high-voltage paper electrophoresis. In this system UDP-[$\text{U-}^{14}\text{C}$]galactose migrated rapidly towards the cathode, separating from the more slowly migrating ^3H -labelled oligosaccharide. The doubly labelled product peak remained on the origin. The incorporation of labelled galactose into oligosaccharide product when [UDP-[$\text{U-}^{14}\text{C}$]galactose and [^3H]oligosaccharide were incubated in the presence of the preparation containing

Fig. 2. Elution of Triton X-100-solubilized rat liver Golgi galactosyltransferases from α -lactalbumin-agarose. An S_{105} preparation (5 ml) was applied and the column (1.8 cm $\times$ 1 cm) of α -lactalbumin-agarose was eluted with 10 mM-Tris/HCl buffer, pH 7.6, containing 10 mM-MgCl₂, 20 mM-KCl, 2 mM-2-mercaptoethanol, 3 mM-N-acetylglucosamine and 1% Triton X-100. The first arrow indicates where N-acetylglucosamine was omitted from elution buffer. The second arrow indicates the elution position of Sigma bovine milk lactose synthetase. Galactosyltransferase activity was measured in the presence of G_{M2} ganglioside (●) acceptor and DSDG fetuin (○) acceptor. Abbreviation: GalT, galactosyltransferase.

Fig. 3. Separation of two G_{M2} ganglioside galactosyltransferase activities by anion-exchange chromatography on DEAE-Sephacrose 6B.

The pooled fraction of G_{M2} ganglioside galactosyltransferase eluted from α -lactalbumin-agarose was applied to the column (9.5 cm $\times$ 2.6 cm) of DEAE-Sephacrose 6B at a flow rate of 15 ml/h. The column was eluted as described in the Materials and methods section of Kaplan & Hechtman (1983). Galactosyltransferase activity was measured in the presence of G_{M2} ganglioside (●) and DSDG fetuin (△). A total of 132 2.5 ml fractions were collected. Protein was determined by the method of Lowry *et al.* (1951) (□). —, Salt gradient.

Table 2. *Acceptor specificity of rat liver Golgi galactosyltransferases after α -lactalbumin-agarose affinity chromatography*
Incubations were as described in the legend to Table 1 and in the Materials and methods section. G_{M2} oligosaccharide was added to a final concentration of 0.26 mM. Abbreviations: N.D., not detected; —, not determined.

Substrate	Activity (nmol/2h per mg)		
	$S_{\alpha Lac}$ preparation	Enzyme $I_{\alpha Lac}$	Enzyme $II_{\alpha Lac}$
G_{M2} ganglioside	4.6	10.6	11.4
Asialo G_{M2} ganglioside	4.4	9.5	10.5
DSDG fetuin	8.3	18.1	6.3
Ovalbumin	3.5	2.2	2.6
DSDG orosomucoid	4.8	5.6	3.8
DS bovine submaxillary mucin	13.3	65.4	22.1
G_{M2} oligosaccharide	—	124.5	160.3
<i>N</i> -Acetylglucosamine	—	N.D.	N.D.
<i>N</i> -Acetylgalactosamine	—	N.D.	N.D.

enzyme $I_{\alpha Lac}$ is indicated by the doubly labelled product peak remaining at the origin. Incubation of enzyme $I_{\alpha Lac}$ with UDP-[U- ^{14}C]galactose in the absence of the oligosaccharide acceptor resulted in the absence of ^{14}C -labelled product at the origin.

Specific activities of galactose transfer to a variety of acceptor substrates catalysed by S_{105} preparations, and DEAE-Sepharose preparations containing enzyme I and enzyme II (Table 1), can be compared with specific activities of galactose transfer catalysed by $S_{\alpha Lac}$ preparations and DEAE-Sepharose preparations containing enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$ (Table 2). The preparations containing enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$ were partially depleted of transferase activities that catalysed addition of galactose to DSDG fetuin, DSDG orosomucoid and ovalbumin (glycoprotein substrates with terminal *N*-acetylglucosaminyl residues). By contrast, galactosyltransferase specific activity towards G_{M2} ganglioside, asialo G_{M2} ganglioside and DS mucin substrates (which have terminal *N*-acetylgalactosaminyl residues) remained unchanged or were increased. Both preparations containing enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$ were 12–15-fold more active towards the G_{M2} oligosaccharide than towards the parent glycolipid substrate. Neither enzyme preparation, however, catalysed disaccharide synthesis.

The results presented in Tables 1 and 2 indicate that, although the affinity adsorbent α -lactalbumin-agarose did remove all of the galactosyltransferase activity towards the monosaccharide acceptors, some residual activity towards the glycoprotein substrates remained associated with G_{M2} ganglioside galactosyltransferase after the two chromatographic procedures. Methods based on comparison of enzymic properties were therefore employed to determine whether the G_{M2} ganglioside galactosyltransferases designated enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$ also catalysed galactose addition to glycoprotein substrates or whether either one or

both of these preparations contained distinct types of galactosyltransferase enzymes with differing acceptor specificities.

Thermolability of galactosyltransferases

Preparations containing enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$ were heated at 37°C for intervals up to 40 min in the absence of substrates, and the rates of thermal inactivation were determined by measurement of enzyme activity in the presence of G_{M2} ganglioside and DSDG fetuin acceptors (Fig. 4). Thermal-inactivation profiles indicating extreme lability were obtained for both enzyme preparations when G_{M2} ganglioside was used as the acceptor substrate, but little or no thermal inactivation of galactosyltransferase activity towards DSDG fetuin occurred. These results suggested that each of the two enzyme preparations contained at least two distinct galactosyltransferase activities. The thermolability of galactosyltransferases measured in the presence of a variety of acceptor substrates is summarized in Table 3. Galactosyltransferase activity towards G_{M2} oligosaccharide was also stable. Activities in both preparations towards two other glycoprotein substrates, DS bovine submaxillary mucin and DSDG orosomucoid, showed thermolability properties that were intermediate between those exhibited by transferases catalysing the addition of galactose to G_{M2} ganglioside and to DSDG fetuin. Although these results are difficult to interpret, this partial thermolability could be explained by overlapping specificities of two galactosyltransferases in each preparation for the last-mentioned two substrates.

Effects of α -lactalbumin on galactosyltransferase activities

The effects of α -lactalbumin on galactose incorporation in the presence of G_{M2} ganglioside, DSDG fetuin and ovalbumin catalysed by preparations containing enzyme $I_{\alpha Lac}$ and enzyme

$II_{\alpha Lac}$ were compared (Fig. 5). The action of this protein effector on a lactose synthase purified from rat liver microsomal fraction has been shown to result in a decrease in the K_m of the enzyme for glucose, thus stimulating lactose synthesis. α -Lactalbumin inhibits the ability of desialodegalacto plasma glycoproteins to serve as acceptors for galactose (Fraser & Mookerjea, 1977). At concen-

trations of 0.125–0.5%, α -lactalbumin stimulated G_{M1} ganglioside synthesis measured in the presence of enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$ (Fig. 6). A

Fig. 4. Rate of thermal inactivation of galactosyltransferases
Enzyme preparations were heated at 37°C in the absence of substrates for the times indicated and assayed at 30°C. Enzyme $I_{\alpha Lac}$: ●, G_{M2} ganglioside substrate; ○, DSDG fetuin substrate. Enzyme $II_{\alpha Lac}$: ■, G_{M2} ganglioside substrate; □, DSDG fetuin substrate. Incubation conditions were as described in the Materials and methods section.

Fig. 5. Effects of α -lactalbumin on galactosyltransferase activities
The effects of α -lactalbumin on galactosyltransferase activities in enzyme $I_{\alpha Lac}$ (—) and enzyme $II_{\alpha Lac}$ (----) towards several acceptor substrates were compared. Incubation conditions were as described in the Materials and methods section except that α -lactalbumin was added at the indicated concentrations. Galactosyltransferase activity was measured in the presence of G_{M2} ganglioside (●), DSDG fetuin (○) and ovalbumin (■) substrates.

Table 3. Thermolability of galactosyltransferases in enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$ preparations
Enzyme preparations were heated at 37°C for 40min in the absence of substrates, after which incubations were performed at 30°C as described in the Materials and methods section.

	Activity remaining after 40min heating at 37°C (%)	
	Enzyme $I_{\alpha Lac}$	Enzyme $II_{\alpha Lac}$
G_{M2} ganglioside	28	26
Asialo G_{M2} ganglioside	30	21
DSDG fetuin	93	106
DSDG orosomucoid	69	62
DS bovine submaxillary mucin	63	60
G_{M2} oligosaccharide	107	109

Fig. 6. Isoelectric focusing of a Triton X-100-solubilized rat liver Golgi preparation (S_{105})

An S_{105} preparation (1 ml) was subjected to flat-bed isoelectric focusing, and enzyme fractions were recovered as described in the Materials and methods section. Galactosyltransferase activity was measured in the presence of G_{M2} ganglioside (○) and DSDG fetuin (●) acceptors. Δ, pH.

maximal stimulation of 1.6-fold was observed for enzyme $I_{\alpha\text{Lac}}$ and of 2.5-fold for enzyme $II_{\alpha\text{Lac}}$. The α -lactalbumin appeared to be acting as a non-specific enzyme-activating agent, since both bovine serum albumin and native fetuin had similar effects when G_{M2} ganglioside was used as acceptor. Addition of α -lactalbumin to reaction mixtures resulted in the inhibition of galactosyltransferase activity in the enzyme $II_{\alpha\text{Lac}}$ preparation towards DSDG fetuin and ovalbumin. Enzyme $I_{\alpha\text{Lac}}$ activity towards ovalbumin was also inhibited, whereas activity towards DSDG fetuin remained unchanged. The profoundly different effects of α -lactalbumin on ganglioside- and glycoprotein-acceptor specific activity in both preparations were again consistent with the idea that two distinct galactosyltransferase enzymes were present in both enzyme- $I_{\alpha\text{Lac}}$ - and enzyme- $II_{\alpha\text{Lac}}$ -containing preparations.

Competition studies

Enzyme preparations were incubated in the presence of UDP-[U- ^{14}C]galactose and two acceptor substrates. The sum of galactose incorporation into reaction products, measured in the presence of G_{M2} ganglioside and a second acceptor substrate individually, was compared with that observed when the two acceptors were added to a single reaction mixture. The results of these experiments (Tables 4 and 5) indicated that only in the presence of both G_{M2} ganglioside and asialo G_{M2} ganglioside was the observed incorporation of labelled

galactose measured with both enzyme preparations significantly different from the calculated theoretical value based on additive incorporation. The glycoprotein and oligosaccharide acceptors did not compete with G_{M2} ganglioside. The G_{M2} oligosaccharide, however, did cause a 40% inhibition of galactose incorporation into acid-precipitable glycoprotein product when incubated together with DSDG fetuin and the preparation containing enzyme $I_{\alpha\text{Lac}}$. The results of the competition analysis confirmed that the G_{M2} ganglioside galactosyltransferase activities were distinct from the glycoprotein-acceptor-specific galactosyltransferases present in both enzyme preparations.

Isoelectric focusing

The results of competition analysis and thermostability studies as well as the different effects of α -lactalbumin on galactosyltransferase activity towards glycolipid and glycoprotein substrates indicate that distinct enzymes catalyse the galactosylation of these substrates. If two such enzymes are not physically associated it should be possible to separate them. The resolution of galactosyltransferases present in the solubilized Golgi preparation (S_{105}) by isoelectric focusing is shown in Fig. 6. Galactosyltransferases in DEAE-Sepharose fractions containing enzyme I and enzyme II were inactivated during isoelectric focusing. G_{M2} ganglioside galactosyltransferase was recovered in two peaks with pI 8.6 and 6.3. DSDG fetuin galactosyltransferase was resolved into three peaks

Table 4. *Competition analysis: Enzyme I_αLac*

Incubations were conducted with the indicated substrates or pairs of substrates. Assay conditions were as described in the legend to Table 1 and in the Materials and methods section, with the exception that DS bovine submaxillary mucin was added at a concentration of 0.25 mg/ml.

Substrates	Activity (nmol/2h)	
	Found	Expected for two enzymes
G _{M2} ganglioside	0.77 ± 0.05	
Asialo G _{M2} ganglioside	0.71 ± 0.02	
G _{M2} ganglioside + asialo G _{M2} ganglioside	0.63 ± 0.02	1.48
G _{M2} ganglioside	0.52 ± 0.01	
DSDG fetuin	1.38 ± 0.14	
G _{M2} ganglioside + DSDG fetuin	1.81 ± 0.05	1.90
G _{M2} ganglioside	1.22 ± 0.08	
Ovalbumin	0.46 ± 0.05	
G _{M2} ganglioside + ovalbumin	1.54 ± 0.01	1.60
G _{M2} ganglioside	1.22 ± 0.08	
DSDG orosomucoid	1.16 ± 0.03	
G _{M2} ganglioside + DSDG orosomucoid	2.19 ± 0.01	2.30
G _{M2} ganglioside	0.75 ± 0.04	
DS bovine submaxillary mucin	1.62 ± 0.10	
G _{M2} ganglioside + DS bovine submaxillary mucin	3.24 ± 0.06	2.37
G _{M2} ganglioside	0.56 ± 0.05	
G _{M2} ganglioside + G _{M2} oligosaccharide	0.58 ± 0.02	0.56
DSDG fetuin	1.65 ± 0.09	
DSDG fetuin + G _{M2} oligosaccharide*	1.00 ± 0.09	1.65

* For which activities are expressed as nmol of acid-precipitable product/2h.

Table 5. *Competition analysis: Enzyme II_αLac*

Incubations were conducted with the indicated substrates or pairs of substrates as described in the legend to Table 4.

Substrates	Activity (nmol/2h)	
	Found	Expected for two enzymes
G _{M2} ganglioside	2.39 ± 0.04	
Asialo G _{M2} ganglioside	2.03 ± 0.10	
G _{M2} ganglioside + Asialo G _{M2} ganglioside	2.09 ± 0.08	4.42
G _{M2} ganglioside	1.43 ± 0.13	
DSDG fetuin	3.03 ± 0.11	
G _{M2} ganglioside + DSDG fetuin	4.57 ± 0.22	4.46
G _{M2} ganglioside	3.14 ± 0.23	
Ovalbumin	2.92 ± 0.03	
G _{M2} ganglioside + ovalbumin	7.77 ± 0.31	6.06
G _{M2} ganglioside	3.14 ± 0.23	
DSDG orosomucoid	3.90 ± 0.02	
G _{M2} ganglioside + DSDG orosomucoid	7.56 ± 0.16	7.04
G _{M2} ganglioside	2.47 ± 0.11	
DS bovine submaxillary mucin	1.74 ± 0.02	
G _{M2} ganglioside + DS bovine submaxillary mucin	3.93 ± 0.17	4.21
G _{M2} ganglioside	1.07 ± 0.08	
G _{M2} ganglioside + G _{M2} oligosaccharide	1.04 ± 0.02	1.07
DSDG fetuin	1.75 ± 0.15	
DSDG fetuin + G _{M2} oligosaccharide*	1.65 ± 0.14	1.75

* For which activities are expressed as nmol of acid-precipitable product/2h.

with pI 8.1, 6.75 and 6.3. Galactosyltransferases with activities towards both G_{M2} ganglioside and DSDG fetuin focused at pH 6.3, suggesting a possible physical association between the two catalytic activities. The two galactosyltransferase activities that focus at the alkaline end of the gradient most probably represent activities present in the enzyme I fraction that did not bind to DEAE-Sephadex at pH 7.6. The galactosyltransferase that focuses at pH 6.75 and manifests very little activity towards G_{M2} ganglioside is considered to represent the glycoprotein-acceptor-specific species that is removed from the S_{105} preparation by α -lactalbumin-agarose affinity chromatography. The use of 50% glycerol in the isoelectric-focusing gel permitted the recovery of 70% of the applied G_{M2} ganglioside galactosyltransferase after isoelectric focusing. Recovery of the glycoprotein galactosyltransferase was 150%.

Discussion

The enzymology of galactose transfer in rat liver Golgi membranes is quite complex. The Golgi extract (S_{105}) contains at least two enzyme species capable of adding galactose to glycolipid acceptors and at least three enzymes that utilize glycoprotein acceptors. Among the goals of the present work has been to achieve the resolution of these activities and to obtain evidence bearing upon possible interrelationships existing among the various galactosyltransferase enzymes of this organelle. The results described in previous work (Hechtman & Kaplan, 1983) provided evidence that two distinct enzymes utilizing G_{M2} ganglioside, with different catalytic properties, could be resolved by ion-exchange chromatography. Evidence cited in the present paper indicates that the solubilized Golgi extract contains, in addition, three activities that catalyse the incorporation of galactose into glycoproteins. One of these activities could be removed from the S_{105} preparation by chromatography on α -lactalbumin-agarose. The remaining galactosyltransferase activity was resolved by anion-exchange chromatography of $S_{\alpha Lac}$ preparation into fractions containing enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$.

The acceptor specificities of galactosyltransferases present in preparations designated enzymes $I_{\alpha Lac}$ and $II_{\alpha Lac}$ were strikingly different from those exhibited by enzymes I and II.

Several reasons can be proposed to account for the broad acceptor specificities of enzyme- $I_{\alpha Lac}$ - and enzyme- $II_{\alpha Lac}$ -containing preparations: (a) distinct transferases in each of the enzyme- $I_{\alpha Lac}$ - and enzyme- $II_{\alpha Lac}$ -containing preparations catalyse the addition of galactose to glycoprotein and glycolipid substrates, but the separation proce-

dures used fail to resolve these enzyme activities; (b) the co-elution of activities in several chromatographic systems could be explained by the presence of a single transferase in each of the enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$ preparations that possesses multiple acceptor specificities; (c) either or both preparations contains two physically associated but functionally distinct galactosyltransferase species, for example a single enzyme protein with two catalytic sites.

The combined results of competition analysis, thermolability studies and the differential effects of α -lactalbumin clearly indicated that either two distinct enzymes or certainly two distinct active sites were present in both enzyme $I_{\alpha Lac}$ and enzyme $II_{\alpha Lac}$ preparations and ruled out the possibility of a single enzyme with multiple acceptor specificity in each preparation. Attempts to resolve galactosyltransferase activities in enzymes $I_{\alpha Lac}$ and $II_{\alpha Lac}$ were only partially successful. Isoelectric focusing of an S_{105} preparation resolved two peaks of G_{M2} ganglioside galactosyltransferase activity and three peaks of DSDG fetuin galactosyltransferase activity. A G_{M2} ganglioside galactosyltransferase that focused at a pH of 8.6 was well separated from a glycoprotein-acceptor-specific galactosyltransferase (pI 8.1). Thus two enzyme activities that were unresolved by chromatography on the α -lactalbumin-agarose and DEAE-Sephadex were shown to be distinct species by isoelectric focusing. Their isoelectric points identify these activities as components of the enzyme-I-containing preparation, since at the pH employed in ion-exchange chromatography (pH 7.6) enzymes with pI values in this range would not be expected to bind to the resin. It did not prove possible to confirm the assignment of the enzymes with pI 8.6 and 8.1 to the enzyme I fraction by direct isoelectric focusing of this preparation owing to its lability to this procedure. An activity that focused at pH 6.75 and had minimal G_{M2} ganglioside galactosyltransferase activity was most probably identical with the activity that was removed from the S_{105} preparation by α -lactalbumin-agarose chromatography.

Two peaks of galactosyltransferase activity, G_{M2} -ganglioside-specific and glycoprotein-specific, co-focused at pH 6.3. These activities most probably represent the galactosyltransferases in enzyme II preparation that bound to anion-exchange resin at pH 7.6. We speculate that the latter two activities, which we have shown to be kinetically distinct but unresolvable by several fractionation procedures, may represent two functionally distinct but physically associated enzyme species.

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References

- Andrews, P. (1970) *FEBS Lett.* **9**, 297-300
- Basu, S., Kaufman, B. & Roseman, S. (1965) *J. Biol. Chem.* **240**, PC4115-PC4117
- Cumar, F. A., Tallman, J. F. & Brady, R. O. (1972) *J. Biol. Chem.* **247**, 2322-2327
- Dicesare, J. L. & Dain, J. A. (1972) *J. Neurochem.* **19**, 403-410
- Esselman, W. J., Laine, R. A. & Sweeley, C. C. (1972) *Methods Enzymol.* **28**, 140-156
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497-509
- Fraser, I. H. & Mookerjee, S. (1977) *Biochem. J.* **164**, 541-547
- Kaplan, F. & Hechtman, P. (1983) *J. Biol. Chem.* **258**, 770-776
- Keenan, T. W., Morré, D. J. & Basu, S. (1974) *J. Biol. Chem.* **249**, 310-315
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Novak, A., Lowden, J. A., Gravel, Y. L. & Wolfe, L. S. (1979) *J. Lipid Res.* **20**, 678-687
- O'Brien, J. S., Norden, A. G. W., Miller, A. L., Frost, R. G. & Kelley, T. E. (1977) *Clin. Genet.* **11**, 171-183
- Schachter, H. & Roseman, S. (1980) in *Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W., ed.), pp. 85-160, Plenum Press, New York
- Schachter, H., McGuire, E. & Roseman, S. (1971) *J. Biol. Chem.* **246**, 5321-5328
- Spiro, R. G. (1964) *J. Biol. Chem.* **239**, 567-573
- Suzuki, Y. & Suzuki, K. (1972) *J. Lipid Res.* **13**, 687-690
- Wang, C. S. & Smith, R. L. (1965) *Anal. Biochem.* **63**, 414-417
- Wolfe, L. S. (1972) in *Research Methods in Neurochemistry* (Marks, N. & Rodnight, R., eds.), pp. 232-248, Plenum Press, New York