Communication

Two Glycosphingolipid Sialyltransferases Are Localized in Different Sub-Golgi Compartments in Rat Liver*

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A highly purified Golgi preparation from rat liver was fractionated on a sucrose density gradient and the activity of two sialyltransferases, CMP-NeuAc: Galβ1→4Glc-Cer (lactosylceramide) α-2→3sialyltransferase; Sat-1), and CMP-NeuAc:Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc-Cer (G_{M1} ganglioside) α2→3sialyltransferase; SAT-4), involved in the biosynthesis of gangliosides were assayed in the collected fractions. These two activities were recovered in different regions of the gradient; SAT-1 was found in a more dense region than SAT-4. This distribution coincided with that of two N-Asn linked oligosaccharide processing enzymes (UDP-GlcNAc:lysosomal enzyme precursor GlcNAc-1-phosphotransferase and UDP-Gal:ovalbumin galactosyltransferase), assumed as putative markers of cis- and trans-Golgi cisternae, respectively. These findings are consistent with the assembly of ganglioside oligosaccharide chains occurring in different sub-Golgi compartments.

The specific localization of glycosyltransferases and other N-Asn-linked oligosaccharide processing enzymes in different compartments of the Golgi stack has been proposed for many cultured cells and solid tissues (1–4). In rat liver the typical morphological arrangement of the Golgi apparatus along the cis-trans axis (5) received further confirmation by compositional (6, 7) and biochemical data (8–10).

Concerning the localization of glycosphingolipid-processing glycosyltransferases, these enzymes are attributed to the Golgi apparatus (11), but their sub-compartmentation has not yet been investigated in any tissue or cultured cell.

In the present work, we fractionated a preparation of highly purified Golgi apparatus cisternae from rat liver on a sucrose density gradient. Throughout the gradient we assayed the activity of two different sialyltransferases (CMP-NeuAc: LacCer[1]α2→3sialyltransferase, SAT-1, and CMP-NeuAc:G_{M1}

α2→3sialyltransferase, SAT-4) involved in the biosynthesis of gangliosides. Comparing the distribution of the above activities with those of two N-Asn-linked oligosaccharide processing enzymes, UDP-GlcNAc:lysosomal enzyme precursor GlcNAc-1-phosphotransferase and UDP-Gal:ovalbumin galactosyltransferase (will be referred to as GlcNAc-1-phosphotransferase and galactosyltransferase, respectively), we wished to elucidate their localization in the different Golgi compartments.

EXPERIMENTAL PROCEDURES

Materials—Dextran (average molecular weight 250,000), 2-mercaptoethanol, α-amylase type X-A from *Aspergillus orizae* and type VIII-A from barley malt, Triton CF-54, ovalbumin (chicken egg albumin grade V), asialofetuin, α-methylmannoside, α-methylgalactoside, and nucleotide sugars were obtained from Sigma. HPTLC plates, sucrose for density gradient ultracentrifugation, and common chemicals were obtained from Merck (Darmstadt, West Germany); *Vibrio cholerae* sialidase from Behringwerke (Marburg, West Germany) and Whatman 3MM paper from Whatman International Ltd (Maldstone, United Kingdom). CMP-N-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid, UDP-[U-^{14}C]galactose, and N-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid were obtained from Amersham International (Amersham, Bucks, United Kingdom); UDP-N-acetyl[6-^{3}H]glucosamine from Du Pont-New England Nuclear.

G_{M1}, G_{M3}, and LacCer (15), [Gal-^{3}H]G_{M1} and other labeled standard gangliosides (16), and [Gal-^{3}H]LacCer (17) were prepared according to the given references.

Subcellular Fractionation—Male Wistar rats (average body weight, 150 g) were purchased from Charles River (Milan). The Golgi apparatus fraction was prepared according to Croze and Morrè (18). About 30 g of fresh tissue was minced and then homogenized in 2 volumes of 37.5 mM Tris maleate buffer, pH 7.0, containing 1% dextran, 0.5 M sucrose, 5.0 mM MgCl$_2$, and 0.1% 2-mercaptoethanol using a Polytron homogenizer (20 ST, Kinematica, Lucerne, Switzerland), 60 s at 8,000 revolutions/min. After centrifugation at 6,000 × g for 15 min, the supernatant was discarded and the upper yellow-brown portion of the pellet was carefully removed with a spatula and resuspended in the homogenization buffer (30–40 ml). Each 10-ml fraction was layered on 25 ml of 1.20 M sucrose solution prepared in the same buffer, covered with cold distilled water, and spun 30 min at 100,000 × g in a Beckman SW-28 rotor; material from the 1.20 M sucrose/homogenate interface was collected with a Pasteur pipette. One-tenth of the collected material was then pelleted (11) to obtain the membranes of the Golgi apparatus fraction. The remaining solution (about 10 ml) was incubated 45 min at 4 °C in the presence of 0.6 mg/ml of each crude amylase (see "Materials"), according to Morrè et al. (7), to promote unstacking of the cisternae. Sucrose was then added to reach a final 50% (w/v) concentration, and 6-ml aliquots were placed on the bottom of two nitrocellulose centrifuge tubes. 4.5-ml fractions of each 45, 40, 35, 30, 25, and 20% (w/v) sucrose solution (density range: 1.17–1.07 g/ml), prepared in 1 mM Tris maleate buffer, pH 7.0, containing 0.1% 2-mercaptoethanol, were overlayered from the bottom to the top, covered with 15% (w/v) sucrose (about 5.5 ml), and the gradient spun at 100,000 × g for 15 h in a Beckmann SW-28 rotor. Twenty-two 1.5-ml fractions were collected by slow aspiration from the top to the bottom of the gradient, diluted with buffer to isotonic solution, pelleted (8), and then resuspended in 0.3–0.5 ml of buffer in order to obtain the enzyme source of the Golgi apparatus subfractions. All procedures were carried out at 4 °C using ice cold solutions.

Enzyme Assays—SAT-1 and SAT-4 activities were determined according to Basu et al. (14). The reaction mixture contained, in a final volume of 0.05 ml, 0.2% Triton CF-54, 0.2 M cacodilate/HCl buffer, pH 6.5, 0.5 mM CMP-[^{14}C]NeuAc (specific activity 1.0 mCi/mmol), enzyme protein (0.5–1.0 mg/ml), and 0.2 mM acceptors LacCer (SAT-1) or G_{M1} (Sat-4). Blanks were regularly prepared by omitting the acceptors in the reaction mixture. Incubation was done at 37 °C for 1 h, and then the reaction was stopped by adding 0.01 ml of

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[1] The abbreviations used are: Glycosphingolipids and gangliosides are coded according to the nomenclature of Svennerholm (12) and the IUPAC-IUB recommendations (13). Designation of glycosphingolipid sialyltransferases (SAT-1 and SAT-4) follows the indications of Basu et al. (14). HPTLC, high performance thin layer chromatography.

chloroform/methanol, 1:1, v/v. The whole mixture was spotted on Whatman 3MM paper and assayed by descending chromatography in 1% tetraborate (14). The radioactivity of the appropriate areas were quantitatively determined by liquid scintillation (using 5 ml of Instagel, Packard) and the blank values subtracted. Galactosyltransferase (19), asialofetuin sialyltransferase (CMP-NeuAc:asialofetuin sialyltransferase) (20), Glc-6-phosphatase (21), 5′-nucleotidase (22), and acid phosphatase (7) were assayed according to the given references. GlcNAc-1-phosphotransferase was assayed according to the procedure of Reitman and Kornfeld (23), using α-methylmannoside as acceptor and UDP-N-acetyl[6-^{3}H]glucosamine as donor (9).

Protein content was determined (24) using bovine serum albumin as the standard.

Reaction Product Characterization—^{14}C-Labeled products, with both LacCer and G_{M1} as acceptors, were eluted from the paper with chloroform/methanol, 2:1, v/v, dried, dialyzed, and lyophilized. The final products before and after *V. cholerae* sialidase action were analyzed by HPTLC and visualized by fluorography, as reported (25).

[^{3}H]Gal products were obtained using cold CMP-NeuAc as a donor and ^{3}H-labeled acceptors (specific activity 20 mCi/mmol, for both LacCer and G_{M1}), following the same conditions described in the regular assays. They were then purified (15) from the radioactive unreacted acceptors and submitted to permethylation. The obtained methylated radioactive galactoses were then analyzed by HPTLC and fluorography (26). Reference 2,4,6-tri-O-methylgalactose was prepared by permethylation of authentic G_{M3} using the same conditions as above.

RESULTS

Reaction Product Characterization—The SAT-1 and SAT-4 activities were determined in rat liver using the total homogenate, the Golgi, and sub-Golgi membrane fractions as the enzyme sources. They were linear in the enzyme protein concentrations and time ranges employed (see "Experimental Procedures").

Using LacCer as acceptor and CMP-[^{14}C]NeuAc as radioactive sugar donor, a radioactive spot was formed that comigrated by HPTLC with the reference G_{M3} and was affected by *V. cholerae* sialidase, liberating radioactive NeuAc. The use of G_{M1} as acceptor provided a radioactive spot, which comigrated with reference G_{D1a} and liberated, as above, radioactive NeuAc upon sialidase action (Fig. 1).

When Gal-^{3}H-labeled LacCer and G_{M1} were used as acceptors and cold NeuAc as sugar donor, the same compounds as above were formed. After purification and permethylation, both provided a radioactive compound that co-migrated by HPTLC with reference 2,4,6-tri-O-methylgalactose, as expected for a galactose C-3 position involved in a glycosidic linkage (data not shown).

Altogether, these findings indicate that the reaction products of SAT-1 and SAT-4 were actually G_{M3} (NeuAcα2→3Galβ1→4Glc-Cer) and G_{D1a} (NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glc-Cer), respectively.

Enzyme Subcellular Distribution—Table I reports the specific activity of both positive and negative marker enzymes in the Golgi apparatus membrane fraction prepared from rat liver, compared with that of SAT-1 and SAT-4. Reliable Golgi apparatus marker enzymes such as asialofetuin sialyltransferase and galactosyltransferase were enriched as much as 60-fold, as were SAT-1 and SAT-4, with respect to the homogenate. Conversely, enzyme markers of other subfractions were only slightly increased or even diminished.

Fig. 2 shows the profiles of protein content, GlcNAc-1-phosphotransferase/galactosyltransferase and SAT-1/SAT-4 activities in the membrane fractions of the sucrose density gradient. Protein content was distributed in a broad peak throughout the gradient. Generally, 35–45% of Golgi protein moved along the gradient, and the others remained associated to the pelleted and unfractionated regions. In the case of transferases, 40–60% of the starting activities were recovered
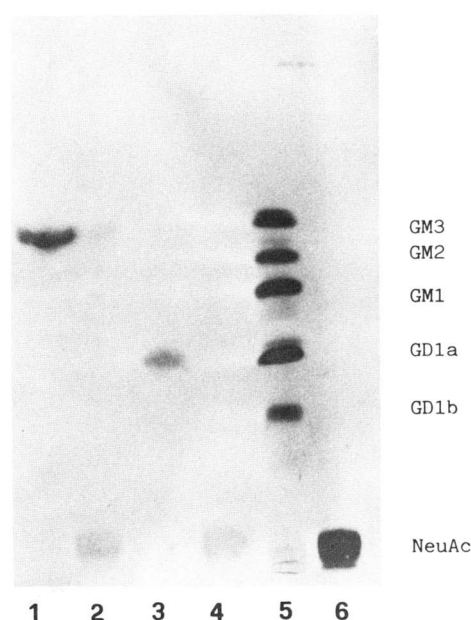

FIG. 1. **Product characterization of SAT-1 and SAT-4 activities using cold acceptors and CMP-[^{14}C]NeuAc as sugar donor.** *Lane 1*, reaction product of SAT-1; *lane 2*, sialidase treatment of lane 1; *lane 3*, reaction product of SAT-4; *lane 4*, sialidase treatment of lane 3; *lane 5*, reference radiolabeled gangliosides; *lane 6*, reference radiolabeled NeuAc. Separation was by HPTLC, using chloroform/methanol/0.2% aqueous CaCl$_2$, 50:42:11, by volume as the eluting solvent system. Revelation was by fluorography.

TABLE I

Activity of SAT-1 and SAT-4 in the Golgi apparatus membrane fraction of rat liver

SAT-1 and SAT-4 have been monitored by measuring the specific activity in the total homogenate and Golgi apparatus membrane fraction (prepared as described under "Experimental Procedures") with respect to the following marker enzymes: asialofetuin sialyltransferase and galactosyltransferase (Golgi apparatus), Glc-6-phosphatase (rough endoplasmic reticulum), 5′-nucleotidase (plasma membranes), and acid phosphatase (lysosomes).

Values are expressed as nanomoles/mg protein/h transferred sugar for all glycosyltransferases and micromoles/mg protein/h released phosphorous for the others. Results are mean values for six experiments. Standard deviations were always less than 10% of the mean value. RSA, relative specific activity; f.t., fresh tissue.

	Total homogenate Specific activity	Golgi apparatus membrane fraction	
		Specific activity	RSA
SAT-1	0.08	4.8	60.0
SAT-4	0.52	32.3	61.5
Asialofetuin sialyltransferase	9.11	534.2	58.6
Galactosyltransferase	1.02	60.2	59.0
Glc-6-phosphatase	6.5	8.4	1.3
5′-Nucleotidase	2.7	5.1	1.9
Acid phosphatase	2.5	1.9	0.8
	mg/g f.t.	mg/g f.t.	
Protein	190.0	0.95	

after fractionation. Notwithstanding some overlapping, these enzymes were distributed in two pairs, GlcNAc-1-phosphotransferase together with SAT-1, and galactosyltransferase together with SAT-4. The former pair, in terms of percentage of recovered activity, showed a maximum at fraction 9 (density 1.13 g/ml), the latter at fraction 11 (density 1.11 g/ml). In the fractions of highest recovery, each of the assayed
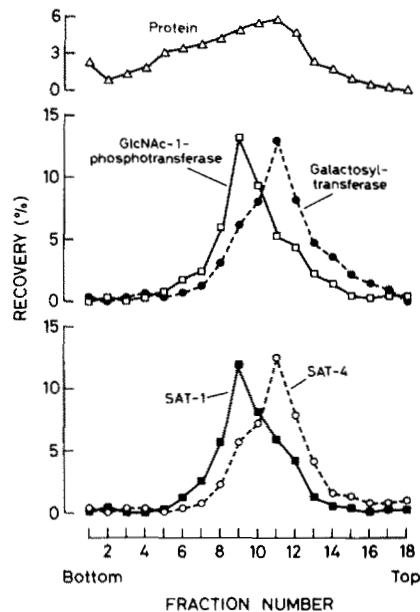
FIG. 2. **Distribution of enzyme activities in the sub-Golgi membrane fractions.** Rat liver Golgi apparatus cisternae have been fractionated by sucrose density gradient as described under "Experimental Procedures." Recovery refers to the enzyme activity in the starting Golgi apparatus membranes. Fractions 19–22 are not shown because no detectable enzyme activity was recorded. For details see text.

enzymes was 2–2.5-fold enriched, in terms of specific activity, with respect to the starting Golgi apparatus membranes. The described profiles refer to the best separation achieved among three distinct experiments. Attempts, in which a continuous or a discontinuous gradient with closer density steps were used, were unsuccessful for a better resolution of enzyme activity.

DISCUSSION

We prepared a highly purified Golgi fraction that was reported to contain intact cisternal stacks (18) and submitted it, after unstacking, to flotation in a sucrose density gradient. The rationale of this approach is that single cisternae can be separated due to the different membrane composition (6, 27).

According to the reported method (18), the Golgi apparatus preparation, after pelleting, was 60-fold enriched in positive marker enzymes (galactosyltransferase and asialofetuin sialyltransferase) with respect to the homogenate. In their turn, SAT-1 and SAT-4 were similarly enriched, as can be expected for Golgi apparatus enzymes (11, 28).

The sucrose density gradient we used follows, in principle, the procedure first described by Knipe *et al.* (29). Adaptation of this method by several authors (8, 30–32) allowed the demonstration of specific sub-Golgi compartmentation of *N*-Asn linked oligosaccharide processing enzymes in different cultured cells and tissues. Following the indication of the cited references, we determined in preliminary experiments the suitable conditions for enzyme resolution. In such a way, we were able to localize, in different density compartments of the Golgi stack, two enzymes, SAT-1 and SAT-4, involved in early (LacCer→G_{M3}) and late (G_{M1}→G_{D1a}) stages of ganglioside biosynthesis. The degree of resolution attained was comparable with that reported for *N*- (8, 30–32) and *O*-linked (33) oligosaccharide-processing enzymes. Because GlcNAc-1-phosphotransferase has been proposed as a putative marker of the cis-face (8, 10, 31) and galactosyltransferase was immunolocalized in the trans-face (34) of the Golgi apparatus,

compartmentation along the cis-trans axis for SAT-1 and SAT-4 can be suggested tentatively.

It should be noted that Rearick *et al.* (35) and Joziasse *et al.* (36), using purified enzyme from porcine submaxillary gland and human placenta, respectively, showed that one activity elaborates the NeuAcα2→3Galβ1→3GalNAc-R sequence of both glycolipids and glycoproteins. Assuming SAT-4 from rat liver exhibits the same property as the above activities, it can be reasonably postulated that there is a crossing point in the oligosaccharide processing of glycolipids and of *O*-linked glycoproteins.

In conclusion, the results of this report present the first direct evidence of a model for ganglioside biosynthesis that involves maturation of the forming oligosaccharide along the different cisternae of the Golgi apparatus. This is in line with previous indirect evidence, mainly based on drug-induced Golgi dissection (37, 38), that similarly suggested a dissociative process for the biosynthesis of gangliosides.

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