Biochimica et Biophysica Acta 814 (1985) 1–7
Elsevier

BBA 72490

UDPglucose-ceramide glucosyltransferase from porcine submaxillary glands is associated with the Golgi apparatus

Hervé Coste, Marie-Bénédicte Martel, Gérard Azzar and René Got *

Laboratoire de Biochimie des Membranes (LBTM-CNRS No. 5421), Université Claude Bernard, Lyon I, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne cedex (France)

(Received July 23rd, 1984)
(Revised manuscript received October 25th, 1984)

Key words: UDPglucose-ceramide glucosyltransferase; Glycolipid; Golgi membrane; (Porcine submaxillary gland)

Subcellular distribution of pig submaxillary gland UDPglucose-ceramide glucosyltransferase (EC 2.4.1.80), the enzyme which catalyses the first step during the sequential addition of carbohydrate moieties for ganglioside biosynthesis, was studied. The results presented strongly suggest that in pig submaxillary gland, the transfer of glucose on endogenous or exogenous ceramides takes place in the Golgi apparatus: the specific activity of UDPglucose-ceramide glucosyltransferase increased in parallel with the activity of a known marker of the Golgi apparatus, UDPgalactose-ovomucoid galactosyltransferase. The specific activity of the glucosyltransferase was 18-times higher in the purified Golgi membranes than in the postnuclear supernatant and the yield was over 30%. An apparent K_m of 22 μM for UDPglucose and 54 μM for ceramides was determined. Maximal glucosylation of endogenous ceramides was achieved at pH 6.5 in the presence of NADH (1 mM) as inhibitor of pyrophosphatases and with Mn^{2+} (5 mM). It was found that the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) is an efficient activator for the glucosylation of exogenous ceramides.

Introduction

Glycolipids appear to be ubiquitous among mammalian tissues, mainly as membrane components. They are synthesized from sugar nucleotides by stepwise transfer of carbohydrates mediated by specific glycosyltransferases [1,2]. It was shown that a number of glycosyltransferases involved in biosynthesis of gangliosides are localized in Golgi fractions isolated from kidney homogenates [3] or from rat liver [4–7]. Enzyme which converts ceramide to galactosylceramide is membrane-bound in kidney, but does not appear to be localized in any single-cell organelle [3]. Less is known about the location of the glucosyltransferase that catalyzes the addition of glucose to the ceramide backbone.

In this report, we present characteristics of the UDPglucose-ceramide glucosyltransferase (EC 2.4.1.80) and evidence that this activity is localized in the Golgi apparatus from porcine submaxillary glands.

Materials and Methods

Materials. Porcine submaxillary glands were obtained from a local slaughterhouse. UDP[U-^{14}C]galactose (309 Ci/mol) and UDP[U-^{14}C]glucose (294 Ci/mol) were from Amersham Interna-

* To whom correspondence should be addressed.

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

tional (Amersham). Unlabelled UDPgalactose and UDPglucose, Mops, Mes, Chaps, phosphatidylcholine from egg yolk, were purchased from Sigma. 2,3-Dimercaptopropanol, also called British Anti-Lewisite was from Serva. Ceramides from bovine brain were purchased from Serdary Research Laboratories, Canada.

Isolation of a Golgi-rich fraction. The subcellular fractionation procedure was a modification of that described by Leelavathi et al. [8]. All subsequent operations were carried out at +4°C. 4 g of minced submaxillary glands were transferred into 20 ml of 0.5 M sucrose and homogenized for 30 s using a Polytron. The homogenate was then centrifuged at $600 \times g$ for 10 min. The resultant postnuclear supernatant was layered on 1.3 M sucrose and centrifuged at $105\,000 \times g$ for 60 min. This resulted in the formation of a clear reddish supernatant, a thick membrane felt above the 1.3 M sucrose interface (smooth microsomes) and a sediment at the bottom of the tube (mitochondria and rough endoplasmic reticulum). The membrane felt was aspirated with the aid of a Pasteur pipette and its molarity was adjusted to 1.1 M sucrose. This suspension was then brought to a volume of 8 ml with 1.1 M sucrose and layered upon an isopycnic sucrose gradient consisting of 12 ml layer of 1.3 M and 6 ml of 1.25 M, followed by 8 ml of 0.5 M sucrose. The gradient was centrifuged 17 h at $105\,000 \times g$. This resulted in two main membrane fractions: one at the interface between 0.5 M sucrose and 1.1 M sucrose (Golgi-rich fraction) and the other at the interface between 1.1 M sucrose and 1.25 M sucrose (smooth endoplasmic reticulum).

Enzyme assays. The glycoprotein β-D-galactosyltransferase (UDPgalactose : 2-acetamido-2-deoxy-D-glucosyl-glycopeptide 4-β-D-galactosyltransferase, EC 2.4.1.38) was assayed according to Fitzgerald et al. [9]. The reaction mixture comprised in a final volume of 260 µl: 55 µM UDP[U-^{14}C]galactose (4.5 mCi/mmol)/30 mM Mops buffer (pH 6.5)/9 mM $MnCl_2$/2.5 mM AMP/0.2% Triton X-100/0.5 mg ovomucoid/30–200 µg protein. Incubation was carried out for 30 min at 32°C: 10 µl were analyzed for substrate integrity as described later and the reaction was stopped by the addition of 2 ml phosphotungstic acid 5% in 2 M HCl. Proteins were collected on glass microfiber filters Whatman (GF/B 2.5 cm) and incorporation of [^{14}C]galactose was determined by liquid-scintillation counting.

The synthesis of glucosylceramide was assayed on endogenous acceptor in a total volume of 110 µl comprising: 50 µM UDP[U-^{14}C]glucose (70 mCi/mmol)/50 mM Mops buffer (pH 6.5)/5 mM $MnCl_2$/2.5 mM $MgCl_2$/1 mM NADH/5 mM dimercaptopropanolol/0.2–0.5 mg protein. For synthesis on exogenous ceramides, the method is adapted from Nescovic et al. [10]. Before incubation, detergent (Triton X-100 or Chaps), phosphatidylcholine and ceramides were dissolved in chloroform/methanol (2 : 1, v/v) at the appropriate concentration and mixed in the incubation tubes. The organic solvent was evaporated under a gentle stream of nitrogen. The same buffer and cofactors as described for endogenous incorporation were added to the dry lipid film as well as the enzymatic fraction and vortexed twice for 30 s. The reaction was then initiated by the addition of 50 µM UDP[U-^{14}C]glucose. Reactions were carried out for 10–120 min at 37°C and was stopped by addition of 2 ml chloroform/methanol (2 : 1, v/v) 1 h at room temperature. The lipid extract was partitioned according to Folch et al. [11]. The lower phase was washed twice with the theoretical upper phase of Folch et al. and dried. Lipids were analyzed as described later; labelled glucosylceramide was localized by autoradiography with LKB Ultrofilm. Radioactive spots were scraped off the plates and counted by liquid scintillation. Upper phases were analyzed by paper chromatography.

Chromatographic methods. Substrate integrity was determined by descending paper chromatography on Whatman 3 MM, developed overnight with ethanol/1 M sodium acetate (pH 3.8) (5 : 2, v/v) as solvent [12]. Glycolipids were analyzed on silica-gel 60 thin-layer plates (Merck) in the solvent system chloroform/methanol/water (60 : 35 : 8, v/v) (solvent A). Borate-impregnated plates were made by immersing precoated silica-gel plates (Merck, Darmstadt, F.R.G.) in a 5% solution of sodium tetraborate in methanol for 1 min. Plates were allowed to dry at room temperature and were activated in the oven at 100°C for 2 h before use. They were developed in chloroform/methanol/water (100 : 30 : 4, v/v) (solvent B).

Chemical determinations. Protein was determined by the method of Lowry et al. [13] modified by Bensadoun and Weinstein [14]. Endogenous free ceramide was measured according to Bouhours and Glickman [15].

Results

Requirements for glucosyltransferase activity on exogenous ceramides

In order to determine the best conditions for glucose incorporation on endogenous ceramides, the influence of different detergents and lipids on the glucosyltransferase activity was assayed (Table I).

Both detergents tested, Triton X-100 and Chaps at a concentration of 5 mg/ml, were largely inhibitors of endogenous transfer and the addition of phosphatidylcholine from egg yolk did not restore the initial activity. Exogenous ceramides (3 mM) gave only a limited activation. The maximal activation was obtained by introducing both Chaps and exogenous ceramides, whereas in the same conditions Triton X-100 was inhibitory. In neither case was the introduction of phosphatidylcholine effective.

TABLE I

REQUIREMENTS FOR THE ACTIVITY OF UDP-GLUCOSE-CERAMIDE GLUCOSYLTRANSFERASE IN THE GOLGI-RICH FRACTION OF PORCINE SUBMAXILLARY GLAND ON EXOGENOUS CERAMIDES

Assay conditions were those described in Materials and Methods. Detergents were added to give a final concentration of 5 mg/ml, phosphatidylcholine was 0.8 mM and ceramides were 3 mM. The amount of protein was 450 μg and the incubation time was 30 min.

Assay conditions	Specific activity (pmol/min per mg protein)
Endogenous activity	6.5
+ Triton X-100	1.0
+ Chaps	1.2
+ Ceramides	9
+ Triton X-100 + phosphatidylcholine	1.4
+ Chaps + phosphatidylcholine	2.2
+ Triton X-100 + ceramides	2.3
+ Chaps + ceramides	15.0
+ Triton X-100 + phosphatidylcholine + ceramides	3.4
+ Chaps + phosphatidylcholine + ceramides	16.0

Identification of products formed by glucosyltransferase

The products of the glucosyltransferase reaction were investigated by chromatography on thin-layer plates developed in two different systems. Fig. 1 shows that the main labelled product in the case of endogenous transfer (more than 95% of the radioactivity from lipid extract) had chromatographic mobility similar to that of monohexosylceramide. On borate-impregnated plates, this lipid was identified as glucosylceramide (Fig. 2). With exogenous ceramides (data not shown), the same R_f values were obtained.

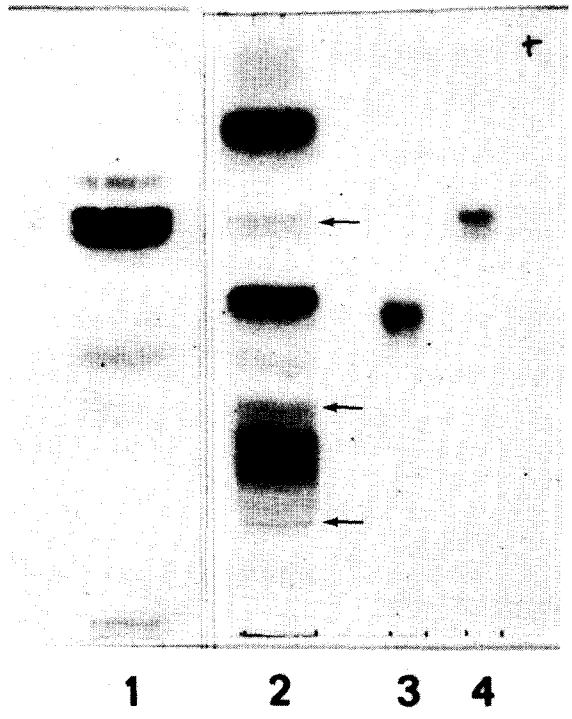

Fig. 1. Thin-layer chromatography of the lipid extract of the Golgi fraction after incubation with UDP[^{14}C]glucose on endogenous ceramides. (1) Autoradiogram of lane 2; (2) lipids of Golgi fraction; (3) cow-milk lactosylceramide; (4) cow-milk glucosylceramide. Thin layer in solvent A, (2-3-4) vizualization α-naphthol/H_2SO_4. Arrows indicate spots with color specific for carbohydrate-containing lipids.

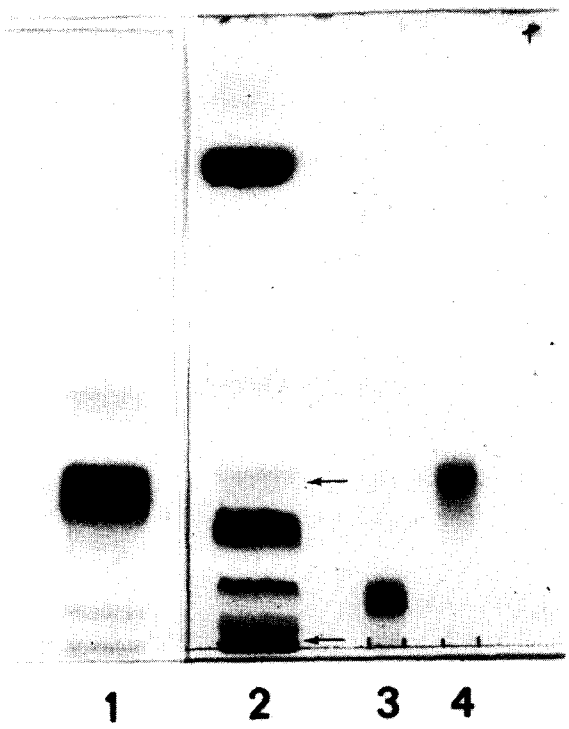

Fig. 2. Chromatography of the lipid extract of the Golgi fraction on borate-impregnated thin-layer plates. (1) Autoradiogram of lane 2; (2) lipids of Golgi fraction; (3) human-milk galactosylceramide; (4) cow-milk glucosylceramide. Thin-layer chromatography in solvent B. Vizualization as in Fig. 1.

Subcellular distribution of enzymatic activities

A comparison of total and specific activities of galactosyltransferase (commonly used as Golgi marker enzyme) and ceramide glucosyltransferase in the various subcellular fractions is shown in Table II. More than 25% of galactosyltransferase total activity was located at the interface between 0.5 and 1.1 M sucrose with an enrichment of 13-fold over the postnuclear supernatant activity. The ceramide glucosyltransferase activity both on endogenous and exogenous ceramides followed the same distribution with a yield of more than 30% in the Golgi-rich fraction and an enrichment of 18-times. These Golgi-derived vesicles were enriched 5.5-fold over postnuclear supernatant in NADPH-cytochrome *c* reductase (5% yield). Vesicle integrity, as determined by latency towards galactosyltransferase [16], was found to be 90%.

Cofactors of glucosyltransferase activity on endogenous ceramides

Some of the requirements for the enzyme reaction are listed in Table III. The measurements of ceramide glucosyltransferase are hampered by the nucleotide pyrophosphatase activity present in the Golgi-enriched fraction [17,18]. The presence of 1 mM NADH was found to be indispensable for a correct determination of the glucosyltransferase. It

TABLE II

GLYCOPROTEIN β-D-GALACTOSYLTRANSFERASE AND UDP-GLUCOSE-CERAMIDE GLUCOSYLTRANSFERASE ACTIVITIES IN SUBCELLULAR FRACTIONS OF PORCINE SUBMAXILLARY GLAND

Results were obtained on one cellular fractionation. Similar specific activities and enrichment were found in three other experiments. Assay conditions were those described in Material and Methods. For glucosylation activity on exogenous ceramides, Chaps was 5 mg/ml and ceramides were 3 mM.

Fractions	Protein in fractions (mg/g gland)	Galactosyltransferase		Glucosyltransferase on endogenous ceramides		Glucosyltransferase on exogenous ceramides	
		total activity (pmol/min per g gland)	specific activity (pmol/min per mg protein)	total activity (pmol/min per g gland)	specific activity (pmol/min per mg protein)	total activity (pmol/min per g gland)	specific activity (pmol/min per mg protein)
Postnuclear supernatant	43.8	2428	55	15.8	0.36	52.6	1.2
Mitochondria, rough endoplasmic reticulum	7.9	646	82	5.9	0.75	24.5	3.1
Smooth microsomes	8.1	1097	135	7.8	0.96	28.3	3.5
Golgi fraction	0.9	630	700	6.1	6.70	20.2	22.4
Smooth endoplasmic reticulum	0.35	88	216	0.7	1.90	2.5	7.1

TABLE III

COFACTORS OF THE ACTIVITY OF UDP-GLUCOSE-CERAMIDE GLUCOSYLTRANSFERASE IN GOLGI-RICH FRACTION OF PORCINE SUBMAXILLARY GLAND ON ENDOGENOUS CERAMIDES

Assay conditions were those described in Materials and Methods. Time of incubation was 30 min. All buffers used were 50 mM (pH 6.5).

Incubation medium	Relative glucosyl-transferase activity (%)	UDPglucose hydrolysis (%)
Complete assay mixture	100	0
– NADH	8	95
– Dimercaptopropanolol	85	0
– NADH, dimercaptopropanolol	5	98
– Mg^{2+}	66	0
– Mn^{2+}	33	0
– Mg^{2+}, Mn^{2+} + 1 mM EDTA	23	0
– Mops + piperazine/glycylglycine	41	18
– Mops + Tris/maleate	48	0
– Mops + cacodylate	13	56
– Mops + Mes	76	0

was shown that after 1 h incubation under standard assay conditions, more than 95% of the original UDP[^{14}C]glucose could be recovered by descending paper chromatography. Whereas the omission of NADH resulted in a loss of more than 90% of transfer activity corresponding to hydrolysis of 95% of the substrate. Dimercaptopropanolol, which is commonly used as inhibitor of pyrophosphatases [19], was ineffective in our hands and so gave little effect on transfer.

Divalent cations were required for maximum activity of endogenous ceramide glucosyltransferase. In the absence of added divalent cations, the reaction proceeded at 66% (minus Mg^{2+}) or 33% (minus Mn^{2+}) of its optimum rate and the addition of 1 mM EDTA resulted in a 75% decrease in activity. The two cations have been included in the incubation medium.

Ceramide glucosyltransferase activity and nucleotide pyrophosphatase inhibition differed noticeably according to the buffer used. In this way, Mops buffer was the best.

The effect of pH is shown in Fig. 3. Maximum activity is observed at pH 6.5 using Mops. There are sharp fall-offs in activity above and below this pH.

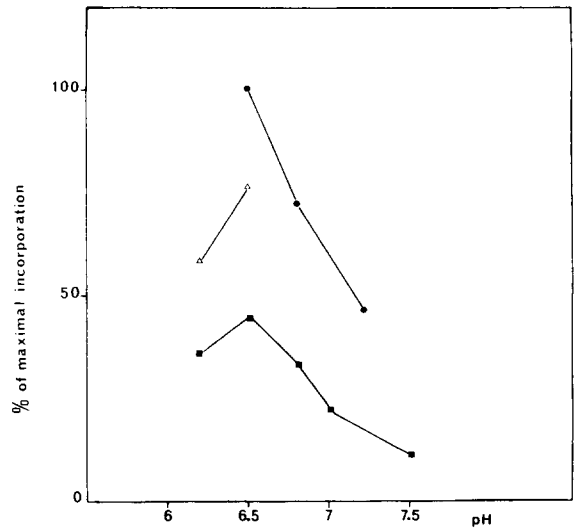

Fig. 3. Effect on pH on the glucosylation of endogenous ceramides from UDPglucose by Golgi fraction from porcine submaxillary gland. ●———●, Mops; △———△, Mes; ■———■, Tris-maleate. In all cases, the final buffer concentrations were 50 mM.

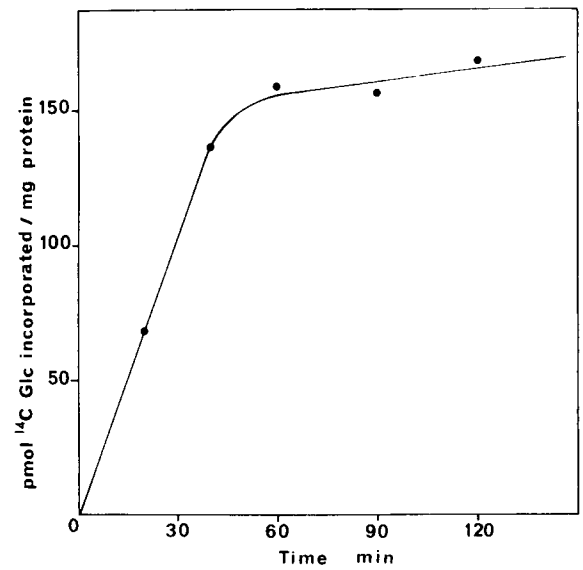

Fig. 4. Effect of incubation time on the rate of glucosylation of endogenous ceramides from UDPglucose by Golgi fraction from porcine submaxillary gland.

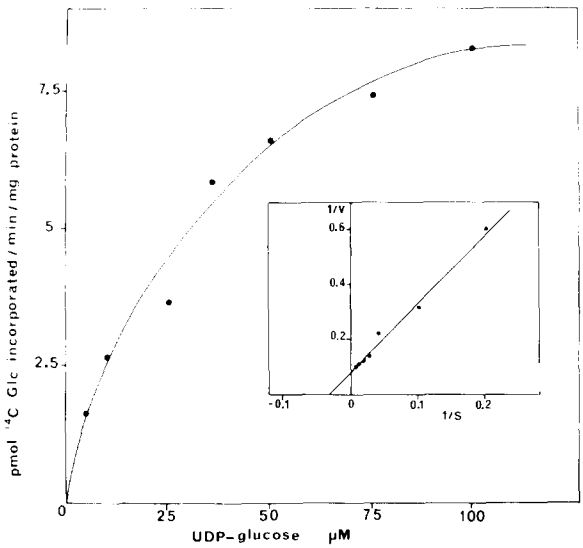

Fig. 5. Effect of different concentrations of UDPglucose on the initial rate of glucosylation of endogenous ceramides by the Golgi fraction. The points of the double-reciprocal plot were fitted by linear-regression analysis ($r^2 = 0.98$) to give a K_m value of 22 μM, and a V_{max} value of 14 pmol/min per mg protein. Units of $1/V$ and $1/S$ (inset) are, respectively, (pmol/min per mg protein)$^{-1}$ and μM^{-1}.

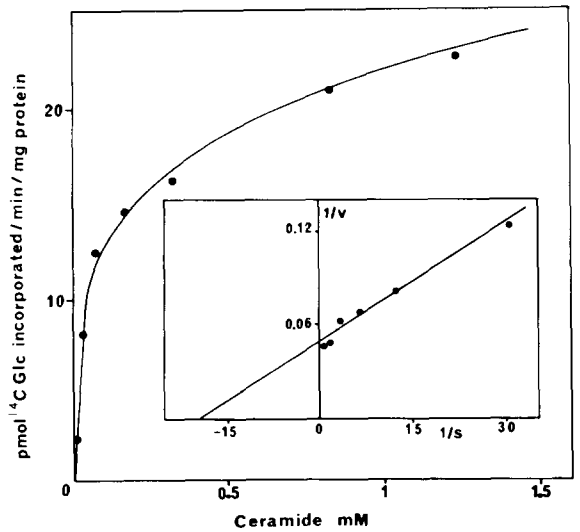

Fig. 6. Effect of different concentrations of exogenous ceramides on the initial rate of UDPglucose-ceramide glucosyltransferase of the Golgi fraction. The points of the double-reciprocal plot were fitted by linear-regression analysis ($R^2 = 0.98$) to give an apparent K_m value of 54 μM and a V_{max} value of 21 pmol/min per mg protein. Units of $1/V$ and $1/S$ (inset) are, respectively, (pmol/min per mg protein)$^{-1}$ and mM^{-1}.

Kinetics of glucose incorporation on endogenous and exogenous ceramides

[^{14}C]Glucose incorporation into endogenous ceramides was first-order with respect to time during incorporation of 40 min (Fig. 4) and with respect to protein concentration up to 0.5 mg per assay. The same results were obtained with exogenous ceramides.

Varying the nucleotide sugar concentration resulted in a typical saturation curve with endogenous ceramides. A Lineweaver-Burk plot of the data yielded an apparent K_m of 22 μM for UDPglucose (Fig. 5).

The effect of adding exogenous ceramide in a concentration varying from 30 μM to 1.5 mM is shown in Fig. 6. The apparent K_m for exogenous ceramides was 54 μM.

Discussion

The data presented provide evidence that the enzyme UDPglucose-ceramide glucosyltransferase is primarily associated with membranes of the Golgi apparatus like other glycosyltransferases involved in the in vitro synthesis of common gangliosides starting from glucosylceramide. It has been shown that ceramide glucosyltransferase from rat brain was localized in smooth microsomes [20]. This was shown in our laboratory to be also true in porcine submaxillary glands. But the subfractionation of the smooth microsomes results in a fraction where the ceramide glucosyltransferase was enriched 7-times. Furthermore, this enzyme copurified with the Golgi apparatus marker enzyme glycopeptide-galactosyltransferase.

These results were obtained first with endogenous ceramides as acceptors for glucosylation. However, incubation of subcellular fractions with exogenous ceramides, in the presence of the detergent Chaps, resulted in the formation of a radioactive glucolipid which comigrated with the same R_f values as the endogenous product in silica-gel thin-layer chromatography. The subcellular distribution of ceramide glucosyltransferase activity was the same as with endogenous acceptor and the Golgi apparatus was implicated as the predominant site of glucosylation. Even though endogenous free ceramide concentration was found to be about 20 nmol/mg protein in Golgi-rich fraction,

a 3-fold stimulation of glucose incorporation was observed with the exogenous acceptor. It was shown that the zwitterionic detergent Chaps was an efficient activator for the glucosylation of exogenous ceramides, whereas this reaction was markedly inhibited by Triton X-100. The glycosylation of endogenous ceramides was also inhibited by these two detergents. It is of interest to note the difference between the glucosyltransferase and the glycosyltransferases involved in the in vitro synthesis of all common gangliosides starting from glucosylceramide: all these enzymes are stimulated by addition of detergent [3–7,21,22]. Similar results have been reported for the UDPgalactose-ceramide galactosyltransferase [23]. On the contrary, treatment of microsomal ceramide glucosyltransferase of rat brain with either Triton X-100 or sodium deoxycholate resulted in almost complete loss of activity [20]. On the basis of this report and the results of our finding, it is tempting to suggest that the ceramide glucosyltransferases have different characteristics than those of the other ganglioside-synthesizing enzymes.

Acknowledgment

We thank Dr. J.F. Bouhours for helping us in the glycolipid analysis.

References

1 Roseman, S. (1970) Chem. Phys. Lipids 5, 270–297
2 Basu, S., Kaufman, B. and Roseman, S. (1973) J. Biol. Chem. 248, 1388–1394
3 Fleischer, B. (1978) in Cell Surface Carbohydrate Chemistry (Harmon, R.E., ed.), pp. 27–47, Academic Press, New York
4 Keenan, T.W., Morré, D.J. and Basu, S. (1974) J. Biol. Chem. 249, 310–315
5 Wilkinson, F.E., Morré, D.J. and Keenan, T.W. (1976) J. Lipid Res. 17, 146–153
6 Richardson, C.L., Keenan, T.W. and Morré, J.D. (1977) Biochim. Biophys. Acta 488, 88–96
7 Yusuf, H.K., Pohlentz, G., Schwarzmann, G. and Sandhoff, K. (1983) Eur. J. Biochem. 134, 47–54
8 Leelavathi, D.E., Estes, L.W., Feingold, D.S. and Lombardi, B. (1970) Biochim. Biophys. Acta 211, 124–138
9 Fitzgerald, D.K., McKenzie, J. and Ebner, K.E. (1971) Biochim. Biophys. Acta 235, 425–428
10 Nescovic, N.M., Sarlieve, L.L. and Mandel, P. (1974) Biochim. Biophys. Acta 334, 309–316
11 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
12 Carminatti, H. and Passeron, S. (1966) Methods Enzymol. 8, 108–111
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
14 Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250
15 Bouhours, J.F. and Glickman, R.M. (1976) Biochim. Biophys. Acta 441, 123–133
16 Fleischer, B. (1981) J. Cell. Biol. 89, 246–255
17 Brandan, E. and Fleischer, B. (1982) Biochemistry 21, 4640–4645
18 Sommers, L.W. and Hirschberg, C.B. (1982) J. Biol. Chem. 257, 10811–10817
19 Faltynek, C.R., Silbert, J.E. and Hof, L. (1981) J. Biol. Chem. 256, 7139–7141
20 Shah, S.N. (1973) Arch. Biochem. Biophys. 159, 143–150
21 Greek, K.E. and Morré, D.J. (1981) Biochim. Biophys. Acta 643, 292–305
22 Senn, H.J., Wagner, M. and Decker, K. (1983) Eur. J. Biochem. 135, 231–236
23 Basu, S., Schultz, A.M., Basu, M. and Roseman, S. (1971) J. Biol. Chem. 246, 4272–4279