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Sphingomyelin is synthesized in the *cis* Golgi

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We have employed in vitro a truncated ceramide analogue with 8 carbon atoms in the sphingosine and the fatty acyl residue, each, to investigate the activity of various membrane fractions to synthesize truncated sphingomyelin. This shortened ceramide readily diffuses through membranes and therefore can easily find access to the lumina of intact organelles. Sphingomyelin synthase activity resides in the Golgi apparatus, and after sucrose density gradient centrifugation of Golgi-enriched fractions sphingomyelin synthesis follows a *cis* Golgi marker enzyme.

Sphingomyelin biosynthesis; Golgi subcompartment

1. INTRODUCTION

Biosynthesis of sphingomyelin is believed to occur in the lumen of an intracellular organelle, because in the plasma membrane of eucaryotic cells this membrane lipid, like glycosphingolipids, is found exclusively in the outer leaflet [1,2]. Several lines of evidence indicate that sphingolipid transport is vesicular and expression on the cell surface occurs by fusion of the vesicles with the plasma membrane. Fluorescent sphingolipid analogues were used to follow this transport, and no sphingolipid-specific flippase has been detected so far that would be required to express on the cell surface any sphingolipid that had travelled through the cytosol by diffusion [3,4].

For our understanding of vesicular transport mechanisms in eucaryotic cells, the knowledge of the exact sites of membrane lipid biosynthesis is a prerequisite. Various intracellular sites and mechanisms of sphingomyelin biosynthesis have been reported in the past [5-7], but more recent evidence suggests the Golgi to be the organelle active in sphingomyelin production [3,4,8]. The Golgi apparatus is divided into *cis*, medial and *trans* cisternae that are mainly discriminated by their individual functions in trimming and processing during glycoprotein sorting and transport (for reviews see [9,10]). More recently, these cisternae have been discussed to be cooperative in the construction of the various oligosaccharides and of glycosphingolipids, as well [11-13]. So far, no differentiation has been reported as to the Golgi subcompartment(s) specifically involved in sphingomyelin biosynthesis.

We have developed a water-soluble, truncated analogue of D-erythro *trans* sphingosine with a chain length of 8 carbon atoms, acylated with [2,3-^{3}H]octanoic acid. This shortened ceramide analogue is designed to penetrate intact biological membranes, and we have employed this amphiphilic substrate to follow sphingomyelin synthesis in vitro of fractions enriched with intact membranes derived from various cell organelles. In the absence of detergent, crude microsomal membranes were active in truncated-sphingomyelin biosynthesis. This has enabled us to detect activity of sphingomyelin synthesis in fractions that were optimally active in a *cis* Golgi marker enzyme.

2. MATERIALS AND METHODS

[2,3-^{3}H]Octanoyl-C_8-D-erythro-sphingosine was prepared according to the concept described in [14] and Karrenbauer et al. (in preparation). The specific radioactivity was 50 Ci/mmol. Cell organelle preparations from rat liver and marker enzyme assays were conducted according to the literature: endoplasmic reticulum and Golgi apparatus (Golgi) [15], and plasma membrane [16]. Partial separation of Golgi cisternae was performed according to [13]. Incubations with truncated ceramide were performed in plastic reaction vials with [^{3}H]t-Ca (in ethanol) pipetted first and dried with a gentle stream of nitrogen. By vigorous shaking with 20 μl TBS, 25 mM EDTA, the radioactive substrate was dissolved and then the membranes (30 μl) were added with gentle shaking. After incubation at 37°C for various times, reactions were stopped by the addition of 1 vol. isopropanol, centrifuged (10000 × *g*, 3 min) and an aliquot of the resulting mixture (usually 5 μl) was analyzed by TLC (Whatman silica gel plates LK6DF) in butanon-2/acetone/H_2O/formic acid = 50:3:5:0.1. The chromatograms were quantitatively evaluated by an automatic TLC-linear analyzer (Tracemaster 20 Berthold). Signal yield was 0.5% of cpm (as determined by liquid scintillation counting) and checked several times by scraping radioactive spots from the TLC-plates, elution of the

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Abbreviations: TLC, thin layer chromatography; t-CA, truncated ceramide C_8C_8; SPH, sphingomyelin; t-SPH, truncated sphingomyelin C_8C_8; CA, ceramide; ER, endoplasmic reticulum; TBS, Tris-buffered saline pH 7.4

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radioactive material and quantitation by liquid scintillation counting. t-SPH synthesis in fractions from sucrose density gradients was assayed as follows: incubations were performed in a final volume of 250 μl, containing 200 μl of sample plus 50 μl of 1 mM Tris-maleate buffer, pH 7.4, 50 mM Na-EDTA, and 1.5 μCi of [^{3}H]t-CA. After incubation for 45 min at 37°C, incubations were stopped by the addition of 5 μl 10% trifluoroacetic acid. These samples were applied to a small column of RP 18 beads (LiChroprep, Merck, about 200 μl bed vol.), equilibrated with 0.1% trifluoroacetic acid in water. The minicolumns were washed with 0.1% trifluoroacetic acid in water (3 ml), and thereafter radioactive material was quantitatively eluted with 1.1 ml of 80% acetonitrile, 0.1% trifluoroacetic acid in water. The eluates were dried in a speed vac concentrator and analyzed by TLC as described. Protein was determined according to [17] after precipitation with trichloroacetic acid (final volume 1 ml, containing 150 μg sodium deoxycholate and 80 mg trichloroacetic acid).

3. RESULTS

3.1. *t-SPH is produced in vitro in intact Golgi membranes*

When [^{3}H]t-CA was incubated with microsomal membranes and the products analyzed by TLC, a result was obtained as shown in fig.1A. Digestion with sphingomyelinase and fast atom bombardment mass spectroscopy have proven that the material of peak 1 represents t-SPH (Karrenbauer et al., in preparation). Peak 2 represents [^{3}H]t-CA. By use of this method, the time dependence of t-SPH formation in Golgi-enriched membrane fractions was investigated. As shown in fig.1B, such intact membranes synthesize [^{3}H]t-SPH linearly with time for about 45 min under the conditions described. This activity corresponds to about 10 pmol t-SPH produced per min and mg of membrane protein. Addition of CDP-choline did not affect this activity in any way. In the presence of 0.1% Triton X-100, no t-SPH synthesis at all could be detected under otherwise identical conditions. To investigate whether SPH synthesis was restricted to Golgi membranes, various organelle preparations were assayed for t-SPH biosynthesis. Less than 5% of the specific t-SPH synthesis activity of Golgi-enriched membranes was found in ER-enriched [15] and plasma membrane-enriched [16] fractions.

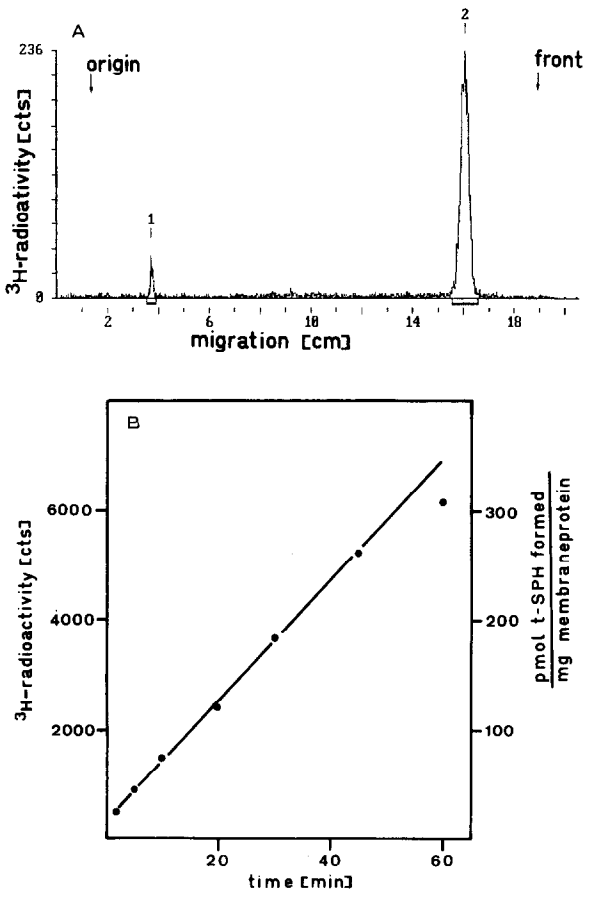

Fig. 1. (A) Radioactivity profile of a chromatogram after incubation of microsomal membranes with [^{3}H]t-CA for 15 min at 37°C and subsequent TLC. (For details see section 2). (B) Time dependence of t-SPH formation in intact Golgi-enriched membranes. Samples were analyzed as shown in fig.1A and as described in section 2.

3.2. *Synthesis of SPH resides in a Golgi fraction characterized by the* cis *Golgi marker UDP-*N*-acetylglucosaminephospho-transferase*

For a further separation of a Golgi-enriched membrane fraction into functionally distinct Golgi subcom-

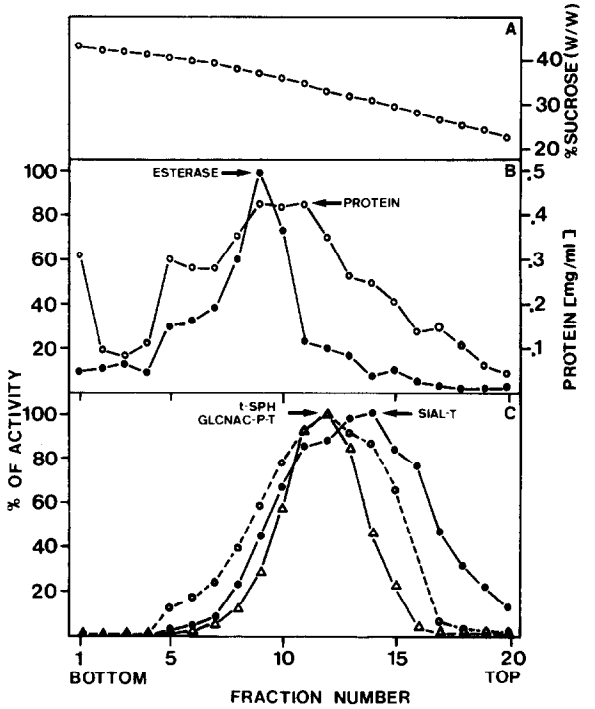

Fig.2. Partial separation of Golgi subcompartments by sucrose density gradient centrifugation according to [13]. (A) sucrose density profile. (B) Protein (○—○) and esterase (●—●) activity profiles. (C) UDP-*N*-acctylglycosaminephospho-transferase (GLCNAC-T) activity (○—○), sialyl-transferase (SIAL-T) activity (●—●), and [^{3}H]t-SPH-formation (Δ—Δ). 26 fractions of 1.6 ml each were collected across the gradient from the bottom. Only the first 20 fractions are depicted here, because only marginal activities of the enzymes investigated were found in the remaining 6 fractions.

partments by sucrose density gradient centrifugation, a combination of procedures was used as has most recently been described [13].

With this method, the authors were able to partially separate *cis* and *trans* Golgi markers and have observed separation of two different sialyltransferases involved in glycosphingolipid synthesis in the fractions characterized by a *cis* and a *trans* Golgi marker, respectively. Following this procedure, we have analyzed the resulting fractions for *cis* and *trans* Golgi marker activities as well as t-SPH synthesis. The result is shown in fig.2C: the two marker enzyme activities could indeed partially be separated from each other and t-SPH synthesis (measured as described in section 2) strictly followed the activity of the *cis* Golgi marker UDP-*N*-acetylglucosaminephospho-transferase. Esterase activity assayed as an ER marker was clearly separated from the optimum of t-SPH formation (fig.2B).

4. DISCUSSION

A truncated ceramide analogue is able to permeate intact biological membranes and therefore allowed us to measure SPH biosynthesis in enriched intact organelles fractions. Remarkably, t-SPH synthesis was completely abolished in the presence of detergent, indicating the need of a high level of structural organization of sphingomyelin synthase for optimal activity. Formation of t-SPH turned out to be independent on added CDP-choline. This finding may be interpreted essentially in two ways: either with the assumption that: (i) biosynthesis of SPH occurs exclusively by transfer of P-choline from CDP-choline to ceramide [18] with sufficient CDP-choline remaining in the isolated membranes; or that (ii) (and more likely), SPH biosynthesis is performed preferentially by transfer of the phosphorylcholine group from phosphatidyl choline to ceramide as has been proposed earlier [7] to occur in the plasma membrane. We are presently reinvestigating these alternatives by use of our t-CA (Schnabel et al., in preparation). The Golgi apparatus was found to be the intracellular site of SPH formation, in line with previous findings [8].

After partial separation of Golgi-enriched membranes into functionally distinct subfractions, we have found that optimal t-SPH synthase activity strictly followed optimal *cis* Golgi marker activity. Although the separation into subcompartments obtained is all but satisfying from a biochemical view, the shift of the marker activities turned out to be reproducible (4 experiments, 8 gradients). Independence of SPH synthesis on added CDP-choline is fortunate from an experimental view, because an artificial activity peak simply caused by the absence of a substrate hydrolyzing activity along the peak fractions does not need to be considered here (a problem that may be inherent to investigations when activated substrates have to be added). Therefore, this data strongly suggests that the *cis* Golgi is indeed the site of SPH formation. This finding will have implications on our future models of biosynthetic vesicular flow through the Golgi apparatus to the plasma membrane and membrane lipid sorting processes involved herein. We are presently studying the kinetics of this flow in vivo by use of the truncated ceramide introduced here. In addition, t-CA may be useful as a competitive in vivo inhibitor of sphingolipid biosynthesis.

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