Sphingomyelin synthase in rat liver nuclear membrane and chromatin

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Abstract The presence of phospholipids in chromatin has been demonstrated, as well as the difference in composition and turnover compared to those present in the nuclear membrane. Recently, some enzymes were also evidenced in chromatin: the base exchange protein complex and neutral sphingomyelinase. The latter has a particular relevance, since sphingomyelin is one of the phospholipids more represented in chromatin. We therefore decided to study the synthesis of sphingomyelin in chromatin and in nuclear membrane isolated from liver nuclei. The evaluation of the enzyme was made (i) using [^{3}H]phosphatidylcholine as donor of radioactive phosphorylcholine and (ii) by identifying the product isolated by thin layer chromatography. In both fractions the enzyme phosphatidylcholine:ceramide phosphocholine transferase or sphingomyelin synthase was present, although with higher activity in nuclear membrane. The enzyme present in the chromatin differs in pH optimum and K_m, showing a higher affinity for the substrates than that of nuclear membrane. The results presented show that sphingomyelin synthase is present not only in the cytoplasm at the level of the Golgi apparatus, but also in the nuclei, at the level of either the nuclear membrane or the chromatin.

Key words: Sphingomyelin; Phosphatidylcholine; Sphingomyelin synthase; Nuclear membrane; Chromatin; Ceramide

1. Introduction

Two pathways for sphingomyelin synthesis are described: the first involves the reaction between CDP choline and *N*-acylsphingosine [1] and the second, described subsequently, consists in phosphocholine transfer from lecithin to ceramide [2]. This reaction is catalysed by the enzyme phosphatidylcholine:ceramide phosphocholine transferase or sphingomyelin synthase (SM synthase), which was found, initially in microsomes of kidney, lung, liver, spleen and heart [3]. In baby hamster kidney (BHK21) cells, the plasma membrane was the subcellular fraction with the highest specific SM synthase activity and, in decreasing order of activity, Golgi apparatus, mitochondria and microsomes [4]. In BHK21 cells, the plasma membrane-located enzyme was responsible for SM synthesis after sphingomyelinase treatment [5]. In Novikoff rat hepatoma cells, prelabeled with [methyl-^{14}C]choline, neophenoxine stimulates the transfer of label from phosphatidylcholine (PC) to sphingomyelin [6]. Recently, it has been demonstrated that the subcellular location of SM synthase is the luminal site of the *cis/medial* Golgi apparatus [7,8], from which SM is transported to the plasma membrane by vesicular flow [9,10]. Brefeldin A, an antibiotic known to cause a dramatic morphological change of the endo-membrane system resulting in a redistribution of Golgi-resident proteins to endoplasmic reticulum [11], causes a dislocation of SM synthase from the Golgi apparatus to the endoplasmic reticulum, where PC and ceramide are synthesised [12], increasing SM synthesis [13].

Since inside the nucleus, associated with chromatin, a phospholipid fraction enriched in SM (35% of that present in the entire nucleus [14]) has been demonstrated, which changes in relation to hepatocyte maturation [15] and proliferation [16], we have supposed an intranuclear SM metabolism. The presence of a neutral sphingomyelinase (N-SMase) in both the nuclear membrane and chromatin has been demonstrated [17]. The SMases present in the two nuclear fractions are characterised by different pH optima. The chromatin SMase activity also changes during liver regeneration [17]. In the present study we have evaluated, in nuclear membrane and chromatin, the possible existence of SM synthase to demonstrate an intranuclear SM cycle.

2. Materials and methods

Radioactive PC (L-3-phosphatidyl-*N*-methyl-^{3}H-choline 1,2-dipalmitoyl, 81.0 Ci/mmol) and Atomlight were obtained from NEN (Boston, MA, USA); PC, non-hydroxy fatty acid ceramide, SM and neutral SMase were from Sigma Chemical Co. (St. Louis, MO, USA)

Thirty-day-old Sprague-Dawley rats of either sex (Harlan Nossan, Milan, Italy) were used. They were kept under a normal light-dark period and had free access to pelleted food and water prior to killing by cervical dislocation after which the livers were quickly removed.

2.1. Isolation of the nuclear membrane fraction

Nuclear membranes were isolated from nuclei according to Kay and Johnston [18].

2.2. Isolation of the chromatin fraction

Chromatin extraction from nuclei was performed according to the method of Shaw and Huang [19] modified by Viola Magni et al. [20].

2.3. Lipid extraction

Lipids were extracted from nuclear membrane (6.2 mg protein/ml) and chromatin (2.5 mg protein/ml) fractions with 20 volumes of chloroform/methanol (2:1 v/v). The organic phase was washed with 0.2 volumes of 0.5% NaCl according to Folch et al. [21].

2.4. Biochemical determinations

Protein, DNA and RNA contents were determined according to Lowry et al. [22], Burton [23] and Schneider [24], respectively. The total amount of phospholipids (PLs) was determined by measuring inorganic phosphorus [25]. Ceramides were isolated with HPLC and measured fluorimetrically after derivatisation according to the method of Rastegar et al. [26] modified by Previati et al. [27].

2.5. SM synthase assays

The SM synthase activity was detected according to the method of Micheli et al. [28]. Reaction mixtures contained 0.1 M Tris-HCl pH

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Abbreviations: PL, phospholipid; SM, sphingomyelin; PC, phosphatidylcholine; PPC, phosphorylcholine; SM synthase, sphingomyelin synthase

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7.6 and 8.4 for nuclear membrane and chromatin respectively, 0.25 mM [^{3}H]PC (final specific radioactivity 1.27 Ci/mol), 2 mM $CaCl_2$, 0.1% Triton X-100 and nuclear membrane or chromatin suspension, equivalent to 100 μg protein, to a final volume of 0.1 ml. Incubation was performed at 37°C for 20 min. The reaction was stopped by adding 2 ml chloroform/methanol (2:1); 0.4 ml of 0.5% NaCl was added to the tubes and vortexed. The tubes were centrifuged at $2000\times g$ for 10 min and the upper phase was removed. The lower phase was dried under nitrogen flow and the lipids were resuspended with chloroform.

2.6. Identification of the product of enzymatic synthesis

The product of the transferase reaction was identified as SM on the basis of its: (a) migration with SM standard on thin-layer silica gels (TLC) chromatograms and (b) degradation by a neutral SMase.

The TLC was made using chloroform/methanol/ammonia (65:25:4 by vol.) as the solvent system [20]. In the sample, 10 μg exogenous SM was added to the tubes before chromatography. The phospholipids were localised with iodine vapour and scraped into counting vials and 10 ml Atomlight were added. In another experiment, the spots corresponding to PC and SM were scraped off, the phospholipids recovered by tree successive elutions with 4 ml chloroform/methanol 2:1 and treated with 0.57 U sphingomyelinase at 37°C for 10 min [28]. Thereafter, the lipids were again extracted and the two fractions, organic and inorganic, were recovered. The organic fraction was again chromatographed in order to verify, after the SMase digestion, the spot which was considered to correspond to SM. The aqueous phase, containing phosphorylcholine (PPC) derived from SM, was used for the determination of radioactivity. The amount of radioactivity present was about 90% of that present in the SM spot before SMase treatment. In order to identify PPC, the aqueous phase, after adding PPC standard, was chromatographed on TLC using chloroform/methanol/ammonia/H_2O (65:25:4:10 by vol.) as the solvent system. The PPC was identified on the basis of its migration related to a PPC standard and was localised with iodine vapour, the PPC spot was scraped into counting vials to which 10 ml Atomlight was added. The amount of radioactivity present was about 85% of that present in the aqueous phase.

2.7. Effect of PC or ceramide concentration on nuclear membrane and chromatin SM synthase activity

Incubations were carried as above reported; in one experiment, the PC concentration was changed between 0.11 and 0.39 mM and between 0.02 and 0.1 mM for nuclear membrane and chromatin respectively by adding exogenous PC; in another experiment the PC concentration was kept constant at a value of 0.11 mM and the ceramide concentration was increased from 0.025 to 0.117 for nuclear membrane and from 0.022 to 0.116 for chromatin by adding exogenous ceramide. In the experiments in which ceramide was added, the presence of a detergent was required: Na-taurodeoxycholate, at a final concentration of 80 μg/ml, was chosen as the most suitable one, according to previous results [4]. The K_m values were determined taking into account the amount of exogenous PC and ceramide added to the samples.

3. Results

The content of DNA, RNA and PLs, in nuclear membrane and chromatin, expressed as μg/mg protein, was similar to that reported previously for 60-day-old animals [15,20]. In the nuclear membrane, the PL content was 117 μg/mg protein (150 nmol/mg protein), of which PC and SM were 60.7% and 3.9% respectively. The content of ceramide was 13.9 nmol/mg protein. In the chromatin, the PL content was 6.11 μg/mg protein (7.8 nmol/mg protein) of which PC and SM were 59.1% and 13.4% respectively, equal to 0.94% and 0.21% of DNA content. Ceramide content was 2.0 nmol/mg protein.

The enzyme had a pH optimum of 7.6 in nuclear membrane and 8.4 in chromatin (Fig. 1a), and its activity was linear in the range from 100 to 400 μg protein (Fig. 1b) and during the first 20 min (Fig. 1c). The enzyme activity obeyed a regular

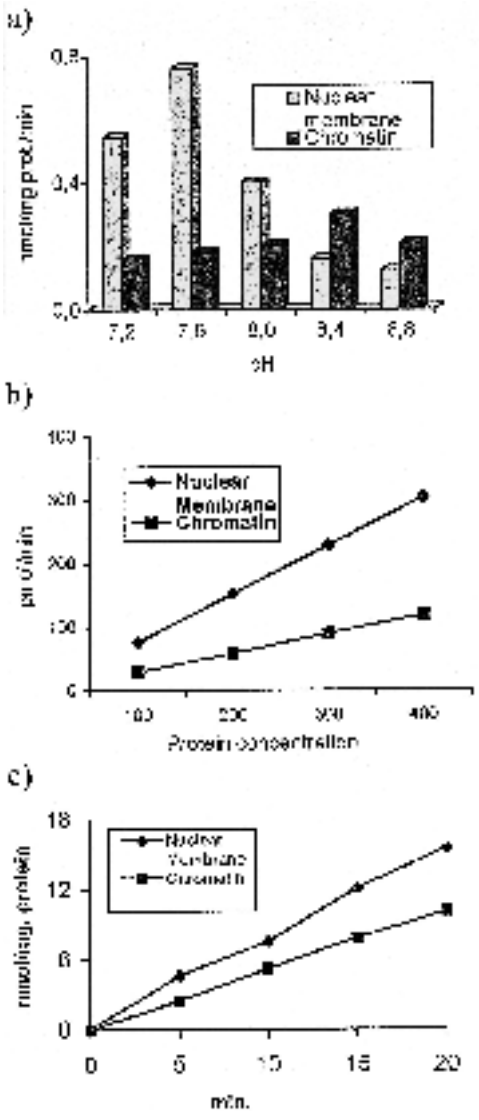

Fig. 1. SM synthase activity in nuclear membrane and chromatin. a: *V*/pH; b: *V*/enzyme; c: *V*/time.

Michaelis-Menten kinetics in both preparations and the K_m values were 1.68×10^{-4} M in the nuclear membrane (Fig. 2c) and 3.59×10^{-5} M in chromatin (Fig. 2a) for [^{3}H]PC (exogenous), and 1.17×10^{-3} M (Fig. 2d) and 2.63×10^{-4} M (Fig. 2b) respectively, for ceramide (exogenous) (see Section 2.7). SM synthase activity was 770 pmol/mg protein/min (V_{max} 1.1 nmol/mg protein/min) in nuclear membrane and 288 pmol/mg protein/min (V_{max} 297 pmol/mg protein/min) in chromatin.

The identification of SM synthesised from [^{3}H]PC was made by TLC, using PC and SM as standards. The results showed the presence of two spots identified as PC and SM (Fig. $3a_2$). After SMase digestion, only one spot corresponding to PC used as donor remained (Fig. $3b_2$) The radioactivity was recovered in the PPC produced by the SMase reaction, as shown by chromatography (Fig. $3c_2$).

4. Discussion

The synthesis of SM is catalysed by SM synthase which transfers PPC from PC to ceramide [2]. It has been shown

that this enzyme is localised mainly in the *cis* and *medial* cisternae of the Golgi apparatus [7,8], although some activity has been found also at the level of the plasma membrane. The ceramide is synthesised in the cytosolic surface of the endoplasmic reticulum and transported to the Golgi apparatus by an unknown mechanism [29,30]. The newly synthesised SM is subsequently transported to the plasma membrane by a vesicular route [9,10]. SM is a PL present in many cell structures including the nuclear membrane and, as recently demonstrated, it is an important component of the chromatin PL fraction [14]. Until now there has been no indication in the literature on how the newly synthesised SM may be transferred to the nuclei and how the SM turnover in this structure is regulated. This aspect appears relevant in view of the fact that diacylglycerol and sphingosine, the latter produced from SM-derived ceramide, regulate the activity of protein kinase C [31], an enzyme which has also been detected in the liver nuclei [32].

The present results show the presence of SM synthase at the level of both nuclear membrane and chromatin in rat liver. The enzyme activity, using labelled ceramide as a substrate, in the liver homogenate is 12.1 pmol/min/mg protein [33]; in the nuclear membrane, we found, with our method, an activity of 770 pmol/min/mg of protein with an enrichment of 64 times, which cannot be explained by possible contaminants, also taking into account differences due to the different assay used. In the chromatin the activity was 288 pmol/min/mg of protein with an enrichment of 24 times with respect to the homogenate. This value is significantly higher than that reported for plasma membranes, using labelled PC as a substrate [4]. The presence of SM synthase in the chromatin cannot be explained by nuclear membrane contamination of the chromatin preparation we used on the basis of specific markers [34] and of chromatin composition [20]. Differences between the two enzymes present in the nuclear membrane and in chromatin have also been found: the optimum pH is 7.6 for the nuclear membrane enzyme and 8.4 for that associated with chromatin; the K_m is 20 times higher for the chromatin enzyme than for that of the nuclear membrane.

It should therefore be concluded that the enzymes present in the nuclear membrane and chromatin are different. This is the first demonstration of a nuclear localisation of SM synthase in rat liver. The different activities found may be tentatively correlated with the SM amount present, which is approximately six times higher in the nuclear membrane than in chromatin.

In a previous work the presence of neutral SMase was also demonstrated in both nuclear membrane and chromatin, in

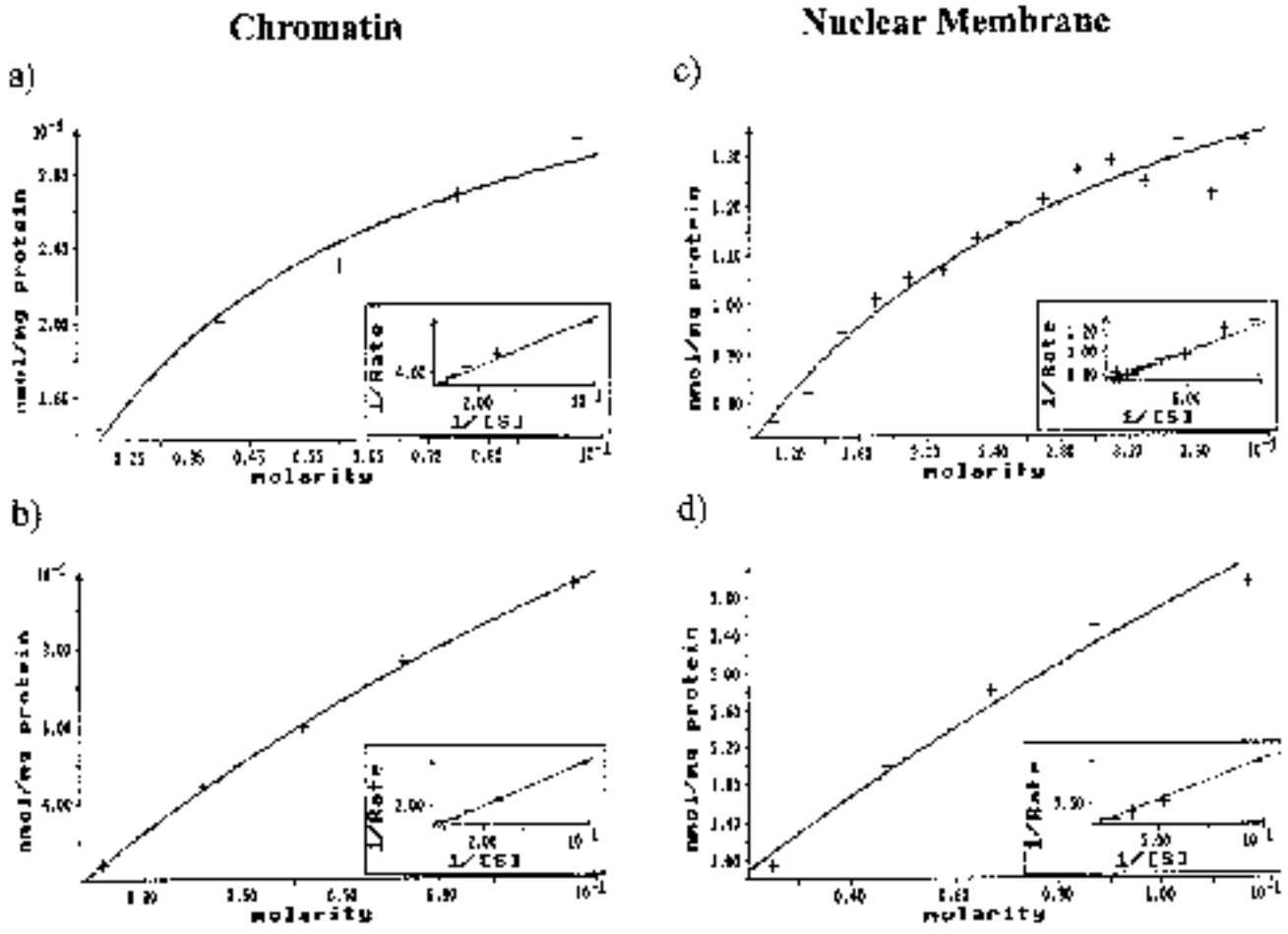

Fig. 2. Effect of PC (a and c) and ceramide (b and d) concentration on nuclear membrane and chromatin SM synthase activity. Incubations were carried at 37°C, pH 7.6 for nuclear membrane and pH 8.4 for chromatin. The results represent the average of two experiments. In the Lineweaver-Burk plots the data are represented as $1/S$ (mM) and $1/V$ (nmol/mg protein/min). The PC and ceramide concentrations were changed by adding exogenous compounds.

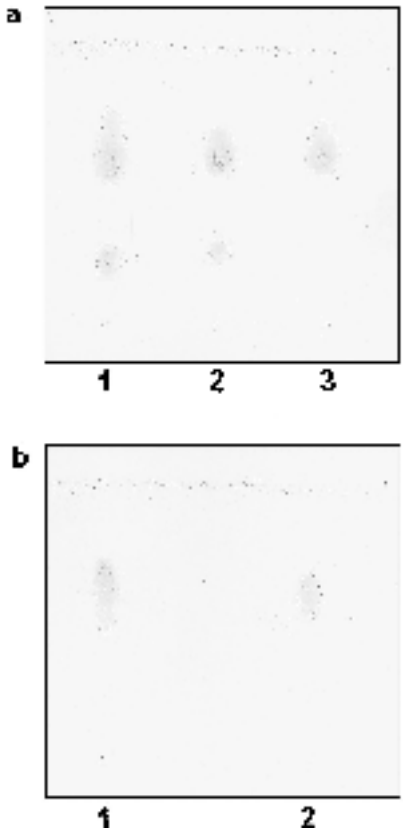

Fig. 3. One-dimensional chromatography on TLC. a_1: PC and SM standard (from the top); a_2: experimental sample; a_3: the same sample after SMase digestion. b_1: PPC standard; b_2: experimental sample. The solvent used was chloroform/methanol/ammonia (65:25:4 by vol.) (a) and chloroform/methanol/ammonia/H_2O (65:25:4:10 by vol.) (b).

this case also the two enzymes seemed to be different on the basis of their catalytic properties [17]. It is evident therefore that synthesis and hydrolysis of SM take place directly at the level of this nuclear structure. In hepatocyte nuclear complex two main PLs are present: PC and SM. Hydrolysis of SM permits the digestion of the residual RNA with RNase, thus suggesting a possible interaction between SM and nuclear RNA [28]. The PC present may represent the source for SM synthesis. SM synthase is present in this complex [28]. Therefore, it appears that the turnover of SM takes place inside the nucleus and the activities of the enzymes are mutually regulated.

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