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# Reverse sphingomyelin-synthase in rat liver chromatin

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**Abstract The chromatin phospholipid fraction is enriched in sphingomyelin content which changes during cell maturation and proliferation. Recently, we have demonstrated that the sphingomyelin variations can be due to chromatin neutral sphingomyelinase and sphingomyelin-synthase activities which differ in pH and $K_m$ optima from those present in nuclear membranes. The sphingomyelin can be used also as a source of phosphorylcholine for phosphatidylcholine synthesis by reverse sphingomyelin-synthase. In the present work we have studied the possible existence of reverse sphingomyelin-synthase activity in nuclear membrane and chromatin. A very low activity was detected in the homogenate, cytosol and nuclear membrane ($0.93 \pm 0.14$, $2.61 \pm 0.33$ and $0.87 \pm 0.13$ pmol/mg protein/min, respectively), whereas the activity present in chromatin was $37.09 \pm 2.05$ pmol/mg protein/min. The reverse sphingomyelin-synthase decreases the intranuclear diacylglycerol pool and increases the intranuclear ceramide pool, whereas sphingomyelin-synthase has an opposite effect. The possible correlation between these enzymes is discussed.**



*Key words:* Chromatin; Phosphatidylcholine; Sphingomyelin; Reverse sphingomyelin-synthase

## 1. Introduction

Phosphatidylcholine (PC) biosynthesis can occur by Kennedy pathway [1], phosphatidylethanolamine (PE) methylation [2], lyso-PC acylation [3], base exchange from phosphatidylserine (PS) [4], and reverse reaction of sphingomyelin-synthase (SM-synthase), which utilizes SM as source of phosphorylcholine (PCh) [5,6]. In cultured normal human skin fibroblasts, after incubation with [$^{14}$C]SM for 17 h, about 65–80% of the cellular radioactivity was recovered in PC [7] and after incubation with [$^{32}$P] or methyl-[$^{3}$H]SM for 20 h, 50% of [$^{3}$H] and [$^{32}$P] was recovered in PC [5]. The concentration ratios PC/SM and diacylglycerol (DAG)/ceramide are intrinsically related, thus providing the cell with a mechanism for the regulation of PC/SM ratio by varying the DAG/ceramide ratio [6]. The reverse reaction of SM-synthase has been localized to the plasma membrane of Ehrlich ascites cells [6] and to the basolateral surface of epithelial cells [8]. Recently, the PC synthesizing enzymes were also described in the nucleus [9–11]. In fact, in several cell types (HepG2, NIH-3T3, and L-cells) the CTP:phosphocholine cytidyltransferase is associated with the nuclear envelope [9]; in CHO cells and hepatocytes the activity of this enzyme was recovered in the nuclear fraction, for 76% and 32%, respectively, [10]. In rat hepatocyte nuclei and isolated nuclear membranes, the choline base exchange activity was also demonstrated [11]. Until now no evidence is available on PC synthesis at the level of chromatin. The chromatin PC and SM in rat hepatocytes differ from those of microsomes and nuclear membranes in turnover [12] and percentage distribution [13]. Changes in the chromatin PC and SM have been observed during cell maturation [14]. The presence in chromatin of some enzymes related to phospholipid (PL) metabolism, namely neutral sphingomyelinase (N-SMase) [15], PC-dependent phospholipase C (PC-PLC) [16] and SM-synthase [17] has been reported. These enzymes can be distinguished from the other present in nuclear membranes on the basis of pH, $K_m$ and $V$/time relationship. The aim of the present study was to investigate the presence of reverse SM-synthase activity in nuclear membrane and chromatin. The results show that this enzyme does occur in chromatin, whereas it is almost undetectable in the nuclear membrane. The presence of SM-synthase and reverse SM-synthase in chromatin may have a relevant influence in regulating the DAG/ceramide ratio at this particular subcellular level.

## 2. Materials and methods

The radioactive SM (choline-methyl-[$^{14}$C], 54.5 Ci/mol) and PC (L-3-phosphatidyl *N*-[$^{3}$H]methyl-choline-1,2 dipalmitoyl, 81.0 Ci/mmol) were obtained from NEN (Boston, MA, USA); Ecoscint A was obtained from National Diagnostic (Atlanta, GA, USA); phenylmethylsulfonyl fluoride (PMSF), PC, SM and PC-PLC were obtained from Sigma (St. Louis, MO, USA).

Thirty-day-old Sprague–Dawley rats of either sex (Harlan Nossan, Milano, Italy) were used. They were kept at 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 and immediately processed.

### 2.1. Preparation of homogenate, cytosol, nuclei, chromatin and nuclear membranes from rat liver

The liver was homogenized in a cold solution of 2.2 M sucrose, 3.3 mM $CaCl_2$ in 1 mM PMSF adjusted to pH 7.2 with concentrated Tris. The hepatocyte nuclei were prepared according to Bresnick et al. [18] in the presence of 1 mM PMSF (Sigma, St. Louis, MO, USA). as described previously [19]. After nuclei isolation, the cytosol was removed and sodium deoxycholate (1% final concentration) was added. The nuclei were then washed twice with Barnes et al. solution [20]. The absence of contamination by the endoplasmic reticulum was shown by electrophoretic analysis of RNA extracted from this preparation [21]. The nuclear pellet still contains some rRNA which completely disappears after two washings in Barnes et al. solution [20].

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*Abbreviations:* SM, sphingomyelin; PC, phosphatidylcholine; PL, phospholipid; DAG, diacylglycerol; SM-synthase, sphingomyelin-synthase

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The nuclei were checked for possible cytoplasmic contamination also by evaluating the activity of a microsomal marker (NADH-cytochrome *c*-reductase) [11]. The swelling of nuclei and chromatin extraction were performed according to the method of Shaw and Huang [22], modified by Viola Magni et al. [19]. Nuclear membranes were isolated according to the procedure of Kay and Johnston [23].

### 2.2. Biochemical determinations

Protein, DNA and RNA contents were determined according to Lowry et al. [24], Burton [25] and Schneider [26], respectively.

### 2.3. Lipid analysis

Lipids were extracted from homogenate, cytosol, nuclear membrane and chromatin 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. [27]. The total amount of PLs was determined, after lipid extraction, by measuring inorganic phosphorus [28]. The SM was isolated by thin layer chromatography (TLC) [19] and determined by measuring inorganic phosphorus [28]. The DAG and ceramide contents were evaluated according to Rastegar et al. [29] and Previati et al. [30], respectively.

### 2.4. Reverse SM-synthase activity assay

The reverse SM-synthase activity was assayed in homogenate, cytosol, nuclear membrane and chromatin. In reaction 1 nmol of [$^{14}$C]SM was diluted by adding 49 nmol cold SM to a final radioactivity of 1.08 Ci/mol.

*Homogenate, cytosol and nuclear membranes*. To establish the optimal conditions of the assay, preliminary experiments were performed changing the SM concentration from 0.05 and 0.6 mM, by the addition of proper amounts of 8μmol/ml exogenous SM, and DAG concentration from 0.05 and 0.6 mM by the addition of proper amount of 6 μmol/ml exogenous DAG, keeping a constant 0.5 mM SM concentration. Also the pH was changed between 7.2 and 8.8. In the optimal conditions, reaction mixture contained 0.1 M Tris–HCl pH 7.6, 0.5 mM [$^{14}$C]SM, 0.5 mM DAG, 6 mM $MgCl_2$, 0.1% Triton X-100 and 100 μg of homogenate, cytosol or nuclear membrane proteins to a final volume of 0.2 ml. Incubation was performed at 37°C for different times until 90 min. The reaction was stopped by adding 2 ml chloroform/methanol (2:1, v/v); 0.4 ml of 0.5% NaCl was added to the tubes under stirring. The tubes were centrifuged at 2000×*g* for 10 min and the upper phase was removed. The lower phase was dried under nitrogen flow, the lipids were resuspended with chloroform and the radioactive PC was evaluated as reported below.

*Chromatin*. To evaluate the optimal conditions of the assay, the SM concentration was changed between 0.02 and 0.4 mM and DAG concentration between 0.02 and 0.3 mM, keeping a constant 0.3 mM SM concentration; the exogenous SM and DAG were added as above reported. Also the pH was changed between 7.2 and 8.8. In the optimal conditions, reaction mixture contained 0.1 M Tris–HCl pH 8.4, 0.3 mM [$^{14}$C]SM, 0.3 mM DAG, 6 mM $MgCl_2$, 0.1% Triton X-100 and 100 μg of chromatin proteins to a final volume of 0.2 ml. The incubation was performed at 37°C for different times until 90 min. Further reaction conditions were the same as those reported above.

*2.4.1. Identification of the product of enzyme action.* The product of the transferase reaction was identified as PC on the basis of: (a) migration using authentic PC as standard by TLC on silica gel 60 (Merck, Darmstadt, Germany) plates and chloroform/methanol/ammonia (65:25:4, v/v/v) as solvent. In the sample, exogenous cold PC was added to the tubes before chromatography in order to identify the spot. The PLs were localized with iodine vapor, scraped off the plates, put into counting vials and dispersed with 10 ml Ecoscint A and 1 ml water. The radioactivity measurements were made with a liquid scintillation analyzer (Packard Instrument Company, Meriden, CT, USA). The radioactivity was recovered only in SM, used as reaction substrate, and PC, product of the reaction. (b) degradation by a specific PLC. In order to evaluate if the radioactivity of the PC spot disappears, the spots, corresponding to PC and SM, were scraped off and the PLs were recovered by tree successive elutions with chloroform/methanol (2:1, v/v). The samples were treated with 1.1 U PC-PLC (Sigma) at 37°C for 15 min. Then, the lipids were again extracted and the two fractions, inorganic and organic, were recovered. The organic fraction was again chromatographed in order to verify the presence of the spot which was considered to correspond to PC. The radioactivity was recovered only in SM. The aqueous fraction was chromatographed in order to evaluate the radioactivity carried by PCh. PCh was identified, comparing with standard PCh, by TLC using chloroform/methanol/ammonia/$H_2O$ (65:25:4:10, v/v/v/v) as the solvent system [16]. The PCh was localized with iodine vapor, scraped off the plates, and counted as described above.

Table 1
Sphingomyelin (SM), ceramide (Cer) and diacylglycerol (DAG) contents and the DAG/ceramide ratio in homogenate (H), cytosol (C), nuclear membrane (NM) and chromatin (Chr)

| | H | C | NM | Chr |
|---|---|---|---|---|
| SM | 6.43 ± 0.36 | 5.27 ± 0.28 | 4.63 ± 0.32 | 1.50 ± 0.27 |
| Cer | 0.20 ± 0.04 | 0.22 ± 0.09 | 1.05 ± 0.03 | 1.99 ± 0.11 |
| DAG | 0.86 ± 0.32 | 0.90 ± 0.49 | 2.03 ± 0.21 | 3.37 ± 0.18 |
| DAG/Cer | 4.3 | 4.1 | 1.92 | 1.69 |

The values are expressed as nmol/mg protein and represent the mean of three experiments ± S.D.

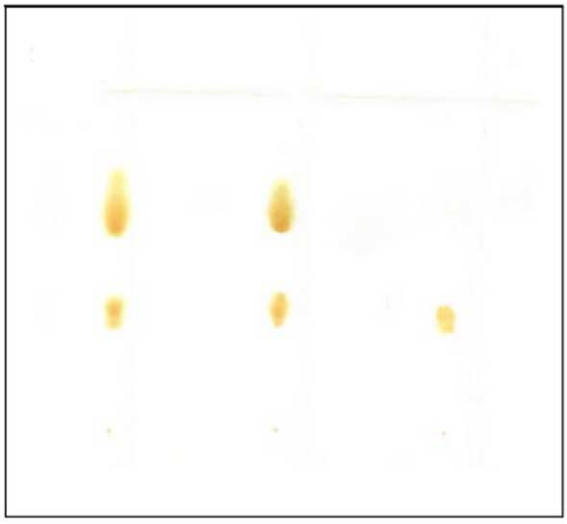


Fig. 1. Identification of the product of reverse SM-synthase. Monodimensional TLC was performed using as solvent chloroform/methanol/ammonia (65:25:4, v/v/v). A: standard PC and SM (from the top); B: incubated sample; exogenous PC was added to the tubes before chromatography to facilitate PC identification; C: the same sample as B after PC-PLC treatment. The PC and SM spots corresponding to PC and SM were scraped, eluted, and treated with 1.1 U PC-PLC at 37°C for 15 min. After lipid extraction, the organic fraction was again chromatographed as above in order to verify the correct identification of PC spot. For details see Section 2.

### 2.5. SM-synthase assay

The SM-synthase activity was detected according to the method of Albi and Viola Magni [17], using [$^3$H]PC as the source of [$^3$H]PCh. The pH of the reaction mixtures was 7.6 for homogenate, cytosol, nuclear membrane, and 8.4 for chromatin.

## 3. Results

The purification level and the composition of nuclear membranes and chromatin were similar to those previously reported for 60-day-old rats [11–13,21]. The SM, DAG and ceramide contents in the different cell fractions are reported in Table 1. In the nuclear membrane, the SM content was 4.63 nmol/mg protein, 77% and 63% of that present in the homogenate and cytosol, respectively; in the chromatin, the value was 1.5 nmol/mg protein. The ceramide and DAG contents were 4.7- and 2.25-fold, respectively in nuclear membrane and

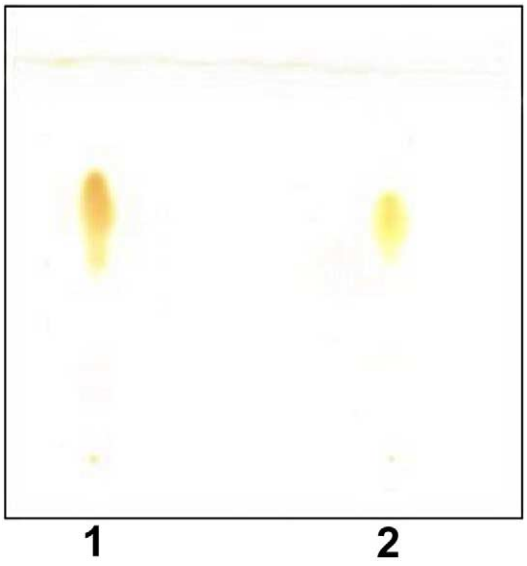


Fig. 2. Identification of PCh. Monodimensional TLC was performed using as solvent chloroform/methanol/ammonia/$H_2O$ (65:25:4:10, v/v/v/v). 1: PCh standard; 2: PCh separated from the sample.

9.5- and 3.7-fold in chromatin than those present in homogenate and cytosol, respectively.

### 3.1. Identification of reverse SM-synthase product

For the identification of reverse SM-synthase product, [$^{14}$C]SM was used as donor substrate and DAG as the acceptor substrate. After the reaction, the PLs present in the incubation medium were separated by monodimensional TLC. Since the chromatin PC, present in 100 μg protein, was 0.3 μg, cold PC was added in order to better identify the spot and the radioactivity was recovered in SM (27.720 cpm) and PC (1386 cpm) spots (Fig. 1A,B). In order to confirm that the PC spot contained really labelled PC, the labelled spots were scraped off, solubilized and digested with PC-PLC. After lipid extraction, the organic phase was again submitted to chromatography where only the radioactive SM spot was present (Fig. 1C), and the aqueous phase showed, after chromatography, radioactive PCh as the only radioactive spot (Fig. 2). Practically, all the radioactivity carried by formed PC was recovered as PCh.

### 3.2. Quantification of reverse SM-synthase activity

The values of reverse SM-synthase activity in the different subcellular fractions are reported in Fig. 3. In homogenate cytosol and nuclear membrane the enzyme activity is extremely low, $0.93 \pm 0.14$, $2.61 \pm 0.33$ and $0.87 \pm 0.13$ pmol/mg protein/min, respectively, whereas in chromatin it is remarkably high, $37.09 \pm 2.05$ pmol/mg protein/min. The enzyme has a pH optimum of 8.4 (Fig. 4a), in agreement with previous observations for other PLs enzymes [18–20]; the reaction is linear with time till at least 90 min (Fig. 4c) and with respect to the protein concentration in the range 50–300 μg (Fig. 4b). In the chromatin, where the DAG content is 3.07 nmol/mg protein, enzymatic activity obeys a regular Michaelis–Menten kinetics and the $K_m$ values are $3.56 \times 10^{-5}$ M for exogenous SM (Fig. 4d) and $1.12 \times 10^{-4}$ M for exogenous DAG (Fig. 4e); the values of $V_{max}$ were 9.5 and 50 pmol/mg protein/min for DAG and SM, respectively.

### 3.3. SM-synthase activity

In homogenate and cytosol the enzymatic activity of SM-synthase is $25.91 \pm 0.06$ and $16.20 \pm 0.13$ pmol/mg protein/min, respectively. In nuclear membranes the enzymatic activity is $770.09 \pm 55.86$ pmol/mg protein/min and in chromatin $278.17 \pm 28.39$ pmol/mg protein/min.

The ratio between SM-synthase and reverse SM-synthase in the different subfractions is reported in Table 2.

## 4. Discussion

This paper provides for the first time evidence for the occurrence of reverse SM-synthase in the chromatin of rat liver. Pagano et al. [31] have shown by using fluorescent method that $C_6$-NBP phosphatidic acid may be converted in the cytoplasm of fibroblast to DAG coupled with CDP-choline for the synthesis of PC. The fluorescent PC labels the plasmatic membranes, and the nuclear membranes, thus confirming that the synthesis of PC in the nuclear membrane is mostly due to the Kennedy pathway [31]. No label was demonstrated inside the nucleus. Our results have shown the presence of PLs in the chromatin, the great part of which is represented by PC. The possibility that PC can be transported by specific protein inside the nucleus cannot be excluded, as well as that other minor pathways may be used for its synthesis. The complex of base exchanges has been detected in nucleus [11]. It has

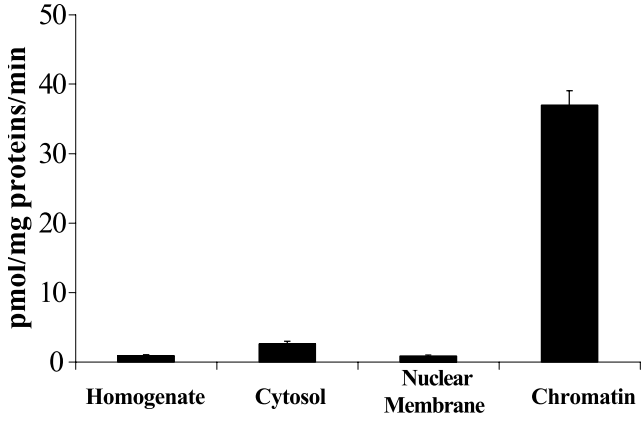


Fig. 3. Reverse SM-synthase activity. The activity of reverse SM-synthase was determined at optimal conditions as reported in Section 2. The data are the mean ± S.D. of tree separate experiments.

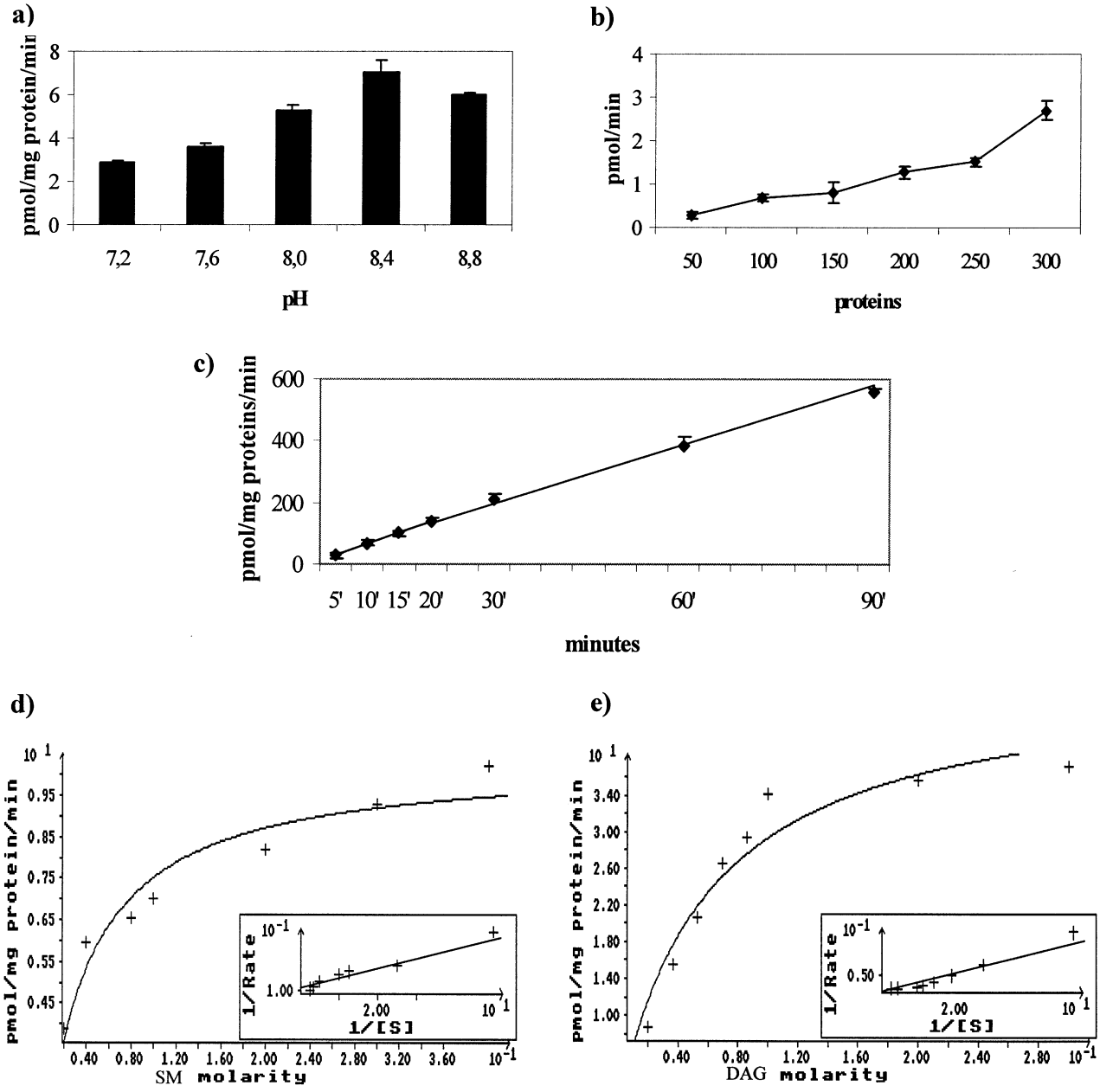


Fig. 4. Reverse SM-synthase activity in the chromatin. a: Effect of pH on the enzyme activity; b: *V*/enzyme course; c: *V*/time course; d: *V*/SM substrate concentration; e: *V*/DAG substrate concentration. The data are the mean ± S.D. of three separate experiments.

been shown that the content of PC may change inside the nucleus in many physiological situations, suggesting some rapid mechanisms with respect to the transport from the cytoplasm. The presence of reverse SM-synthase may induce quick modifications of intranuclear ceramide and DAG contents in relation to the enzyme activity which may be counterbalanced by the SM-synthase activity. Actually there is no sufficient information in order to establish if SM-synthase and reverse SM-synthase are the same enzyme or two different enzymes. It must be taken into account that SM-synthase activity in the chromatin is 7.5 times higher than that of reverse SM-synthase. This ratio increases in nuclear membrane in which the activity of reverse SM-synthase is very low, thus suggesting that the PC synthesis may be due to the other enzymatic reaction. This difference may have influence on the DAG/ceramide ratio, which is lower in chromatin, thus suggesting a possible utilization of DAG for PC-synthesis.

Many roles have been attributed to ceramide, either in cell growth and differentiation [32,33] or in apoptotic processes [34]. Most of these researches concerns the cellular pathways

Table 2
The SM-synthase/reverse SM-synthase ratio in homogenate (H), cytosol (C), nuclear membrane (NM) and chromatin (Chr)

| | H | C | NM | Chr |
|---|---|---|---|---|
| SM-synthase/reverse SM-synthase | 27.86 | 6.20 | 885.05 | 7.49 |

of SM and the increase of cytosolic ceramide. The target of this second messenger is considered to be the protein kinase C, which is activated by DAG and inhibited by ceramide and sphingosine [35]. This effect is evident on cytosolic kinase, whereas an opposite effect is observed on the protein kinase associated to the membranes, as shown in cells treated with TNFα [36]. Recently an increase in ceramide was observed in hepatocyte nuclei after lobular ligation [37], a situation which induces apoptotic modifications of hepatocytes. In our case it cannot be excluded that the intranuclear ceramide increase blocks the cells in G1 by inhibiting enzymes, such as DNA primase, or favoring the aggregation of chromatin as an initial apoptotic event. On the other hand, it may be equally supposed that the activation of nuclear membrane kinase by ceramide may favor the phosphorylation of phosphoproteins related to the cell cycle, thus favoring cell proliferation.

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