Nuclear sphingomyelin protects RNA from RNase action

Marta Micheli[a], Elisabetta Albi[a], Claude Leray[b], Mariapia Viola Magni[a,*]

[a]*Istituto di Patologia Generale Università degli Studi di Perugia, Policlinico Monteluce, Perugia, Italy*
[b]*ETS Strasbourg 10, rue Spielmann, 67065 Strasbourg Cedex, France*

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Abstract Chromatin phospholipidic fraction, as previously demonstrated, shows the same localization as RNA inside the nuclei. DNase and RNase treatment of nuclei removed almost totally the DNA, 63% of RNA and caused a 50% loss of phospholipids. The aim of the present investigation is to study the fraction of RNase undigested nuclear RNA and its relationship with the phospholipids still present in the nuclei. Isolated hepatocyte nuclei were treated with Triton X-100 and digested with RNase and DNase. The undigested nuclear material contained proteins (98%) and a small amount of RNA (1.7%), DNA (0.4%) and phospholipids (0.18%). The analysis of phospholipids showed the presence of two components only, namely phosphatidylcholine and sphingomyelin. In the same complex, the activity of sphingomyelin synthase, phosphatidylcholine-dependent phospholipase C and neutral sphingomyelinase has been detected. Treatment of isolated RNA with neutral sphingomyelinase modified the RNA in RNase sensitive RNA, thus suggesting that the SM may represent a bridge between two RNA strands possibly regulating transcription.

Key words: Sphingomyelin; Phosphatidylcholine; Sphingomyelin synthase; Phosphatidylcholine-dependent phospholipase C; Sphingomyelinase; Double-stranded RNA

1. Introduction

The presence of phospholipids (PLs) associated with the chromatin has been demonstrated with histochemical and biochemical techniques [1–3]. This chromatin fraction was different in composition and turnover from that present in nuclear membranes and microsomes [4,5]. The synthesis of PLs increased in relation to DNA synthesis during liver regeneration [6] and their composition changed in association with the maturation process [7]. Localization of the PLs inside the nuclei was similar to that of RNA, as demonstrated by using double labelling with phospholipase A2-gold and ribonuclease-gold [8]; in fact, both electrondense granules were present in the border zone between hetero- and euchromatin, in the interchromatin granules and in the nucleolus [8]. The relationship RNA-PLs was confirmed by biochemical analysis. In fact, nuclei and nuclear membranes lose PLs after RNase digestion, whereas no loss after enzymatic digestion with DNase was observed [9]. Nevertheless, also after prolonged digestion with DNase and RNase, a small part of RNA was RNase resistant; this RNA is generally indicated as double-stranded RNA (dsRNA) [10]. No evidence exists so far for a possible association between PLs and dsRNA. The aim of this work is to establish the interactions of nuclear PLs, in particular SM, with RNA.

The results show that two phospholipids, sphingomyelin (SM) and phosphatidylcholine (PC) are present in the complex together with the enzymes sphingomyelin synthase (SM synthase), phosphatidylcholine-dependent phospholipase C (PC-PLC) and neutral sphingomyelinase (N-SMase).

After digestion of SM, RNA becomes RNase sensitive, thus suggesting that SM may represent a bridge between two RNA strands protecting them from the RNase action.

2. Materials and methods

DNase I, SMase, SM and PC were purchased from Sigma Chemical Co. (St. Louis, MI, USA); RNase cocktail (RNase A = 10 μg/ml and RNase T1 = 200 U/ml) from Ambion (Austin, TX, USA); [colinemethyl-^{14}C] sphingomyelin (*bovine*) (54.5 Ci/mol) and L-3-phosphatidyl[*N*-methyl-^{3}H] coline,1,2-dipalmitoyl (81.0 Ci/mmol) were obtained from Amersham (Buckinghamshire, England, UK).

Sprague-Dawley male and female rats 60 days old (Harlan Nossan, Milano, Italy), fed ad libitum and kept at a normal light-dark period, were used for the experiments. Under ether anaesthesia the animals were laparatomized and the liver was perfused with cold 0.25 M sucrose solution pH 7.2.

2.1. Hepatocyte nuclei preparation

The hepatocyte nuclei were prepared according to Bresnick et al. [11] as modified by Viola-Magni et al. [3].

2.2. Triton X-100 nuclei (NT)

After two washings in 40 vol. of TMS (10 mM Tris-HCl, pH 7.5; 2.5 mM $MgCl_2$; 0.5 mM PMSF (phenyl-methyl-sulfonyl fluoride) and 0.25 M sucrose) nuclei were incubated with 1% Triton X-100 in TMS for 10 min at 0°C, in order to remove the external nuclear membrane [12], and centrifuged at 1000×*g*. for 15 min. The pellet was washed twice with 40 vol. of TMS [13].

2.3. Triton X-100 nuclei digestion (DNT)

Triton X-100 nuclei, suspended in Barnes et al. solution [14], were digested according to Hermann et al. [15], with DNase I (120 μg/ml) and Rnase cocktail for 15 min at 37°C. The digested nuclei were sedimented at 800×*g* for 5 min, suspended in 0.4 M $(NH_4)_2SO_4$, 30 mM Tris, pH 7.4. and pelleted at 10000×*g* for 15 min [15]. The pellet, re-suspended in Barnes solution [14], was dialysed against 200 vol. of Tris-HCl 0.1 M at pH 8.4 [16].

2.4. Biochemical determinations

DNA [17], RNA [18], and protein [19] contents were determined. The total lipids were extracted with 20 vol. chloroform:methanol (2:1 v/v) according to Folch et al. [20] and the total amount of PLs was determined by inorganic phosphorous measurement [21].

*Corresponding author. Fax: +39 (75) 5722194.
E-mail: ispatgen@unipg.it

Abbreviations: DAG, diacylglycerol; DNT, digested nuclei after Triton X-100 treatment; EDTA, ethylendiaminetetra-acetic acid; NT, nuclei after Triton X-100 treatment; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-dependent phospholipase C; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLs, phospholipids; PS, phosphatidylserine; SM, sphingomyelin; N-SMase, neutral sphingomyelinase; SM synthase, sphingomyelin synthase; TLC, thin layer chromatography; Tris, hydroxymethylaminomethane

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2.5. Lipid analysis

The single PLs were separated by TLC in a bidimensional system and identified with markers [7]; the spots were then scraped and the amount of each PL was evaluated by measuring the inorganic phosphorus [21]. Hydrolysis of SM was made according to Sweeley and Moscatelli [22] using 2 N HCl in aqueous methanol at 75°C for 5 h. After removing fatty acids by a hexane wash, the lower phase was alkalinized with 7 N NaOH and the sphingoid bases were extracted by mixing with diethyl ether. Sphingoid bases were derivatized with biphenylcarbonyl chloride according to Jungalwala et al. [23]. Sphingoid derivatives were separated on a 120×4.6 mm ID HPLC column packed with 5 μm LiChrsorb RP 18 (Merk) with methanol/water (95:5 v/v) as a mobile phase (flow rate: 1 ml/min). Detection was carried out with an ultraviolet detector (UV-975 Jasco) at 280 nm. Standard sphingosine was used for identification and quantitative evaluation. The fatty acids composition of PL complex, separated by TLC in a unidimensional system, was analyzed according to Leray et al. [24].

2.6. RNA isolation

RNA was extracted from DNT according to Chirgwing et al. [25] and isolated according to Sambrook et al. [26]. The RNA obtained, free of contaminant since it was treated during the isolation with protease, chloroform and methanol, was RNase resistant as confirmed by RNase cocktail treatment.

2.7. Enzyme assay

The SMase activity was detected according to the method of Albi et al. [27]. The method of Schultze et al. [28] was employed for PC-PLC assay. The labelled ^{3}H PC was diluted with cold PC to a final specific activity 1.27 Ci/mol. Reaction mixtures contained 0.1 M Tris/HCl pH 8.4, 0.125 mM ^{3}H PC, 2 mM $CaCl_2$, 0.1% Triton X-100 and sample suspension equivalent to 100 μg protein to a final volume of 0.2 ml. Incubation was performed at 37°C for 15, 30, 45, 60, 90, 120 and 150 min. The reaction was stopped by adding 4 ml chloroform/methanol (2:1), 0.8 ml of 0.5% NaCl was added to the tubes and mixed with vortex. The tubes were centrifuged at 2000×*g* for 10 min, the upper phases were removed and diluted in counting vials with 10 ml Atomlight.

The same reaction mixture described for PC-PLC was used for SM synthase assay and the radioactive SM was measured in the lower phase. The phase was evaporated under nitrogen flow. Then 1 ml of chloroform containing cold SM was added to the tubes for re-suspending the lipids before chromatographic analysis. Monodimensional separation on TLC was made by using chloroform/methanol/27% ammonia (65:25:4 by vol.) as solvent. The PLs were localized with iodine vapour and scraped into counting vials to which 10 ml Atomlight was added. The identification of PLs was made by using commercial PLs as standard. The radioactivity was recovered only in SM and PC.

The optimal conditions of enzymatic assay (pH, V/time, V/enzyme and V/substrate concentration) were chosen on the basis of previous analysis made for both enzymes on isolated chromatin.

2.8. RNA digestion

(a) Samples of 25 μg of isolated RNase insensitive RNA, isolated from DNT nuclei, were dissolved in Tris-EDTA 10 mM pH 8.4 and were incubated for 5 min at the following temperatures: 37°C, 65°C and 95°C. The samples were immediately cooled in ice and digested with RNase cocktail for 30 min at 37°C. The reaction was stopped in ice and the amount of RNA, precipitated with absolute ethanol, was evaluated. (b) Samples of 25 μg of RNA, isolated and purified as previously described (see Section 2.6) were incubated with 0.1 M Tris-HCl pH 8.4, 6 mM $MgCl_2$, 0.1% Triton X-100 in the presence of 5 μl (6 U/mg prot) of N-SMase. The RNA was incubated at 37°C. After 60 min RNase cocktail was added and the reaction was carried out for a further 30 min. The reaction was stopped in ice and the amount of RNA, precipitated with absolute ethanol, was evaluated and compared with that of samples incubated without SMase.

3. Results

3.1. Chemical composition

Hepatocyte nuclei (HN), nuclei after Triton X-100 treatment (NT) and digested nuclei after Triton X-100 treatment (DNT) were analyzed for their content in proteins, DNA, RNA and PLs (Table 1). The data, referred to the weight of fresh liver, showed that the proteins recovered from HN represent approximately 3‰ of the liver weight and this is consistent with the fact that the hepatocytes in the adult rat liver constitute 45–55% of the cellular population [29] and that the nuclear proteins are less than 1% with respect to the total cell mass [30]. The Triton X-100 treatment caused a 22% loss of protein which was probably due to Triton X-100 which destroys most of the cytoplasmic surface of nuclear membrane (Table 1). The DNT were particularly rich in protein since 1/3 of the nuclear content remained in the undigested material. As expected, the DNA amount in the HN remained unmodified after Triton X-100 treatment (Table 1). After enzymatic digestion an almost undetectable fraction, corresponding to less than 1% of the pre-existing nuclear DNA, remained (Table 1). It can be concluded that DNT contained insignificant amounts of DNA. The RNA amount in the HN was 126 μg/g of liver weight, equal to 20% of DNA. Triton X-100 treatment caused a 23% RNA loss, probably a fraction linked to the nuclear membrane which was damaged by Triton X-100 treatment. After enzymatic digestion 16% of the total nuclear RNA, which is insensitive to DNase-RNase treatment, remained (Table 1). The amount of PLs, evaluated as inorganic phosphorus, in the HN was 72 μg/g of liver weight and a strong decrease was observed after Triton X-100 treatment, the remnant was only 19%, a small part of which (3%) remained in the DNT (Table 1).

Therefore, the DNT is constituted by a large amount of proteins, undigested RNA after RNase treatment, a small fraction of PLs and less than 1% of nuclear DNA.

3.2. Phospholipid

The bidimensional chromatographic separation of PLs HN and NT showed the presence of PC, phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS) and SM (Fig. 1a). As expected, in the HN, PC was the most represented and its percentage was 60%, followed by PE (21%), PI (12%), PS (3.5%) and SM (3.9%, Fig. 2a). There

Table 1
Differences in protein, DNA, RNA and PL composition of isolated hepatocyte nuclei (HN), in nuclei after Triton X-100 treatment (NT) and in NT after DNase and RNase treatment (DNT)

	HN	NT	DNT
Protein	3100.00 ± 141.42 (100%)	2233.00 ± 125.83 (78%)	1183.11 ± 193.65 (38%)
DNA	622.00 ± 42.57 (100%)	615.00 ± 39.69 (98%)	4.45 ± 2.45 (0.7%)
RNA	126.00 ± 16.23 (100%)	97.50 ± 3.75 (77%)	20.88 ± 6.47 (16%)
PLs	71.65 ± 9.38 (100%)	13.30 ± 3.82 (19%)	2.14 ± 0.34 (3%)

The values are expressed in μg/g of liver weight and as % reduction relative to HN. Each value represents the mean of five experiments ± S.D. PLs, phospholipids.

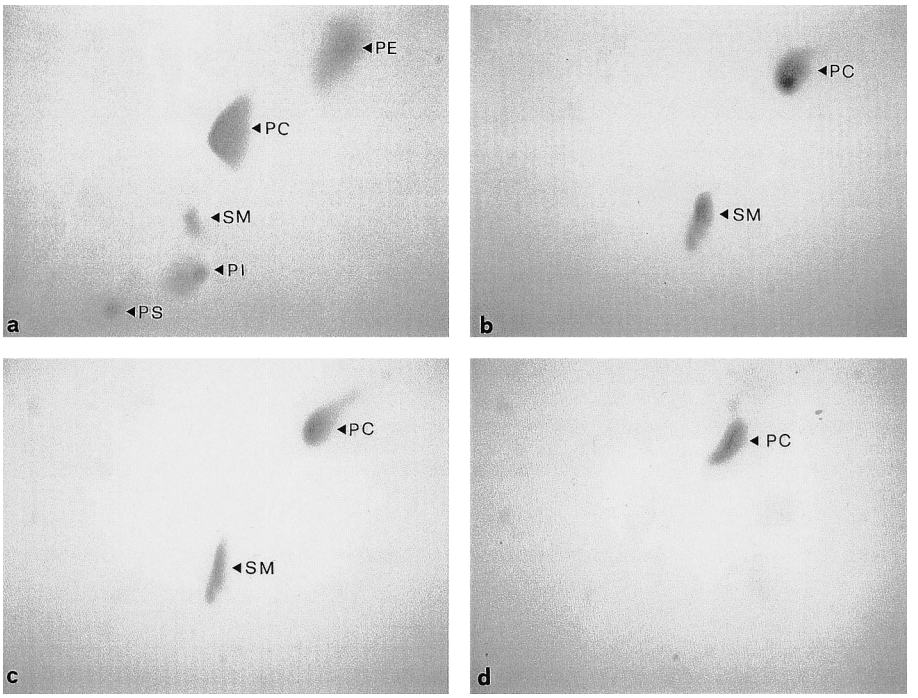

Fig. 1. Bidimensional chromatography on TLC system: a: nuclei PLs; from the top in the following order: PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine. b: PC and SM markers; from the top in the following order: PC, SM. c: DNT PLs; from the top in the following order: PC, SM. d: DNT PLs after SMase digestion; the spot corresponding to PC.

was no significant difference between the PL composition in HN and NT, also if there was a strong decrease (81%) in PL due to Triton X-100 treatment as described above. The PL of DNT showed after chromatography only two spots, which can be identified as PC and SM on the basis of the markers (Fig. 1b, c). The two spots were also similar in size: in fact, the quantitative analysis showed that PC represents 52% and SM 48% of the total DNT PLs. Considering the nuclear PL composition, the PC content was 60% of the total nuclear PLs, corresponding (according to our data) to 42.9 μg/g of liver weight. Since the PLs of the DNT were approximately 3% of those of the nuclei, equivalent to 2.1 μg/g of liver, it can be calculated that the PC present in the DNT is 1.2 μg. The total nuclear SM is 2.7 μg/g of liver weight, one third of this amount (equal to 0.9 μg) is present in the DNT. The amount of PC in DNT is not dissimilar to that found in the nuclei, whereas the SM appears strongly concentrated in DNT PLs. In order to confirm that the spots localized in the lower part of chromatography were really SM, the scraped spots, after solubilization, were digested with N-SMase and again chromatographically separated. The chromatography showed only one spot corresponding to PC (Fig. 1d). Further confirmation was obtained by sphingosine determination after hydrolysis of SM. The value of sphingosine was 0.24 μg/g of liver. Since the SM value is 2.5 times higher than that of sphingosine, the value of SM resulted equal to 0.6 μg/g of liver; considering that the recovery was around 70–80%, a value very near to that calculated on the basis of inorganic phosphorous determination was obtained. The analysis of fatty acid composition of PC and SM did not show any consistent difference with respect to the chromatin PC and SM, thus indicating that these PLs were in the intranuclear structure (Table 2).

Table 2
Fatty acid composition of PC and SM present in DNT

Fatty acid	PC	SM
16:0	34.27 ± 4.24	36.57 ± 6.28
18:0	18.96 ± 4.01	14.89 ± 2.72
18:1n-9	13.10 ± 3.40	14.35 ± 4.51
18:2n-6	8.77 ± 3.60	9.97 ± 7.20
20:4n-6	10.03 ± 1.19	4.15 ± 3.67
22:6n-3	4.00 ± 2.23	–
Sat./unsat.	0.8 ± 0.17	0.51 ± 0.31

Values are expressed as mole percentage of total fatty acid methyl esters; only major components are listed. Each value is the mean of three experiments ± S.D.
PC, phosphatidylcholine; SM, sphingomyelin; DNT, nuclei after Triton X-100 treatment and after DNase and RNase treatment.

3.3. Enzymatic activity

The enzymes PC-PLC, SM synthase and SMase already described in the chromatin were present in DNT. In previous investigations it was found that optimal pH was 8.4 for all enzymes, V/enzyme was linear from 50 μg to 1500 μg of protein content and the K_m was 8×10^{-5} M for PC-PLC, 3.6×10^{-5} M for SM synthase and 2.4×10^{-5} M for SMase. The temperature used for the assay was 37°C.

The enzymatic activities, referred to the RNA present, were similar in HN and NT (Table 3). In DNT, the PC-PLC shows a modest increase, the SM synthase value almost doubles and N-SMase is 4 times more active, thus indicating that the two enzymes, related to SM metabolism and especially N-SMase, may have a specific role in DNT. This conclusion was confirmed when the enzymatic activity was referred to mg protein. The enzyme PC-PLC decreased by 23% in NT and by 50% in DNT with respect to the HN value, thus suggesting that a great amount of PC-PLC is concentrated in the nuclear

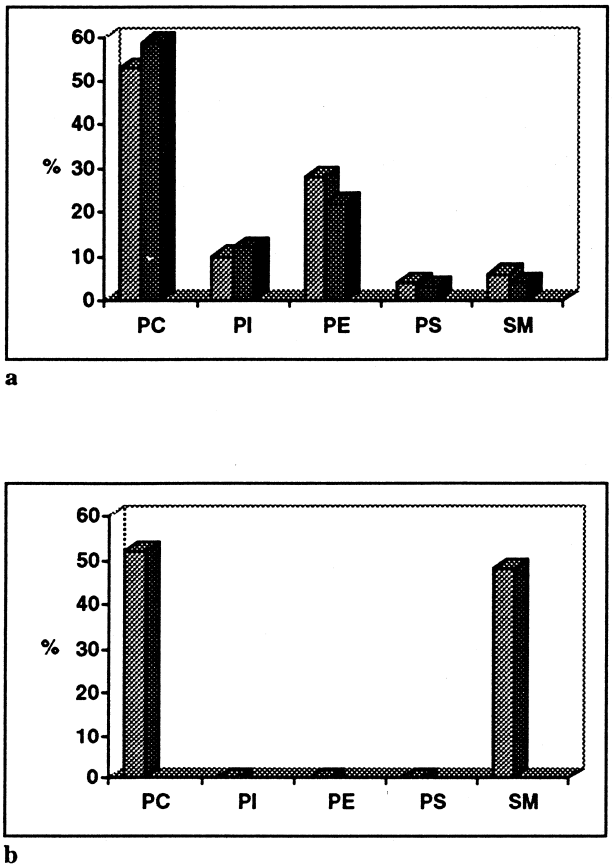

Fig. 2. Percentage distribution of single PLs in: a: isolated hepatocyte nuclei (HN), in nuclei after Triton X-100 treatment (NT) and in b: NT after DNase and RNase treatment (DNT). PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

membrane or in the Triton X-100 nuclei before DNase and RNase treatment. No variations were observed for SM synthase, whereas the N-SMase increased reaching a value in DNT which is almost twice that of HN, thus confirming the role of this enzyme in the DNT (Table 3).

3.4. RNA

The RNase resistant RNA, isolated from DNT, became sensitive to enzymatic hydrolysis when it was submitted to thermal denaturation. The digested RNA was 10% and 25% at 37°C and 65°C respectively. The amount increased sharply after incubation at 95°C, at which temperature 90% of undigested RNA was hydrolysed by RNase. The same result was obtained when the RNA was incubated with N-SMase, also in this case 90% became sensitive to RNase treatment. This finding demonstrates that the SM is strongly bound to RNA and cannot be solubilized by the solvent treatment used during RNA extraction.

4. Discussion

Previous results showed that enzymatic digestion of RNA causes a loss of PLs in the hepatocyte nuclei [9]. Nevertheless, a fraction of RNA cannot be hydrolysed even by DNase-RNase treatment thus suggesting the presence of dsRNA. In order to analyse this RNA and to see its relationship with chromatin PLs, the isolated hepatocyte nuclei were treated with Triton X-100 and digested with DNase and RNase cocktail. At the end a complex remained formed by protein, undigested RNA, PLs and an insignificant amount of DNA. The analysis of PLs showed that only two PLs are present, PC and SM, which are the most polar among the PLs. The percentage of SM is equal to that of PC, whereas in the entire nuclei SM is only 3.9% of the total PLs, 1/5 with respect to PC. The presence of SM in DNT was confirmed by the evaluation of sphingosine. The PLs present in the complex show the same fatty acid composition as those isolated from the chromatin, so it is possible to suppose that they do not make part of nuclear membrane but that, particularly for SM, we are dealing with the form bound to a carrier protein. It can be suggested, therefore, that the isolated complex represents a specific structure.

If this conclusion is correct, it is necessary to see its possible role in the nuclear function. The analysis of PL enzyme activities evidentiates the presence of PC-PLC, SM synthase and N-SMase [27], already described in isolated chromatin. Following the hydrolysis of SM by SMase, the RNA becomes RNase sensitive. This is the first demonstration of a direct interaction between RNA and SM, indicating a possible role of PLs inside of the nuclei. The RNase resistant RNA becomes sensitive after heat treatment, as happens for dsRNA already described in the nucleus [10], as well as after SMase digestion. This result may suggest an interaction between the SM and the two RNA strands, which is destroyed by the high temperature. It is possible that phosphorylcholine, present in SM, represents a bridge between two RNA strands. The N-SMase present in the complex, whose activity, expressed for mg protein, is higher in the DNT, destroys this bridge and the RNA becomes single stranded. The activity of N-SMase can be regulated by PC-PLC, although the mechanism is not yet clear. In fact, it has been demonstrated that DAG, which is produced after the hydrolysis of PC, stimulates the activity of acid SMase [30], but it seems to have no influence on N-SMase. It must also be considered that the reported evidences

Table 3
Neutral sphingomyelinase (N-SMase), phosphatidylcholine phospholipase C-dependent (PC-PLC) and sphingomyelin synthase (SM synthase) activities in isolated hepatocyte nuclei (HN), in nuclei after Triton X-100 treatment (NT) and in NT after DNase and RNase treatment (DNT)

	HN	NT	DNT
N-SMase	0.30 ± 0.10	0.36 ± 0.10	1.31 ± 0.16
	(13.1 ± 4.38)	(17.38 ± 4.86)	(22.15 ± 2.7)
PC-PLC	4.18 ± 1.00	3.04 ± 0.26	5.88 ± 0.67
	(180.0 ± 0.04)	(140.0 ± 0.01)	(93.13 ± 8.83)
SM synthase	0.37 ± 0.04	0.27 ± 0.08	0.6 ± 0.01
	(15.83 ± 1.97)	(13.05 ± 3.72)	(10.82 ± 0.39)

Values are expressed as nmol/mg RNA/min and, in parenthesis, nmol/mg protein/min. Each value represents the mean of three experiments ± S.D

concern the cytoplasmic N-SMase and there is no information about the nuclear form. The decrease in SM, following its hydrolysis, is compensated by SM synthesis throughout SM synthase, which utilizes PC as donor of phosphorylcholine. It is therefore clear that the presence of these two PLs and the enzymes (PC-PLC, SM synthase and N-SMase) have a precise significance. PC represents the source of SM and at the same time its hydrolysis by PC-PLC influences the SMase activity. The SM is linked to RNA and causes its insensitivity to RNase. The regulation of this system via the enzymes modifies therefore the RNA by exposing it to the action of RNase. The RNA present in the complex behaves under this aspect as dsRNA. DsRNA was described first in infected cells, and more recently also in normal cells including hepatocytes [31]. Many functions are attributed to dsRNA such as the regulation of transcription as a system of anchorage [32]. Recently, it has been shown that dsRNA is able to activate a protein kinase which inhibits the synthesis of IF2α [33]. At the moment there is no evidence about the nature and the function of RNA linked to SM and therefore the exact significance of the presence of SM in the regulation of nuclear RNA cannot be understood. It may be possible to hypothesize a role of PL in nuclear RNA maturation, particularly in the displaying process by linking the RNA expressed by intron and blocking their transfer to the cytoplasm. After SM digestion this RNA will be hydrolysed by RNase.

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