Sphingomyelin Synthase, a Potential Regulator of Intracellular Levels of Ceramide and Diacylglycerol during SV40 Transformation

DOES SPHINGOMYELIN SYNTHASE ACCOUNT FOR THE PUTATIVE PHOSPHATIDYLCHOLINE-SPECIFIC PHOSPHOLIPASE C?*

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Sphingomyelin synthase (SMS), an enzyme involved in sphingomyelin (SM) and ceramide metabolism, can potentially regulate, in opposite directions, the levels of ceramide and diacylglycerol. In this study SMS activity was investigated in normal and SV40-transformed human lung fibroblasts (W138). The addition of [3H]C2-ceramide to cells resulted in a time-dependent formation of [3H]C2-SM. At 24 h after treatment, normal W138 cells cleared 17% of [3H]C2-ceramide producing [3H]C2-SM, which accounted for 13% of total radioactivity. On the other hand, SV40-transformed cells cleared 45% of [3H]C2-ceramide and produced C2-SM, which accounted for 24% of total radioactivity. This enhanced production of C2-SM was also supported by an increase in the total SMS activity of cells (measured in vitro), such that SV40-transformed cells had SMS activity of 222 pmol/mg of protein/h, whereas wild type cells had 78 pmol/mg of protein/h of activity. Additional studies aimed at examining the SMS activity directed at ceramide produced in the plasma membrane. Treatment of cells with exogenous bacterial sphingomyelinase (SMase) for 25 min resulted in cleavage of 90–95% of total SM and the concomitant generation of ceramide. After bacterial SMase treatment, wild type W138 cells cleared ceramide very slowly (19.2 pmol of ceramide/mmol of phospholipid P1 after 6 h of incubation) and hardly regenerated any SM. On the other hand, SV40-transformed cells cleared ceramide much faster (41.1 pmol/mmol of P1 after 6 h of incubation) and regenerated approximately 80% of the original SM. These results show that the enhanced SMS activity of transformed cells is particularly pronounced when ceramide is produced in the plasma membrane.

Finally, several observations led us to consider the relationship of SMS to the “putative” phosphatidylcholine-specific phospholipase C (PC-PLC). We, therefore, tested the effects of D609, a purported PC-PLC-specific inhibitor on the activity of SMS. D609 inhibited SMS activity in vitro. In addition, cellular studies showed that SMS activity was dramatically inhibited by concentrations of D609 used previously to study PC-PLC (10–50 μg/ml). These results suggest SMS as an important biochemical target for D609, and they raise the distinct possibility that many of the roles of PC-PLC, especially in cell transformation, may be attributable to SMS.

Lipid molecules play a central role in signal transduction and regulation of cell growth and viability. Indeed, it is now becoming obvious that the biological relevance of lipid molecules transcends their structural functions as components of cellular organelles, as it has been shown that lipids also exert an active role in the complex network of reactions that regulate cellular physiology. The concept of lipids as signaling and regulatory factors was first applied to the glycerophospholipids and the products of their metabolism. Thus, molecules such as diacylglycerol (DAG),1 inositol trisphosphate, arachidonate, phosphatidate, and platelet-activating factor have been associated with the regulation of basic events that control intracellular homeostasis and intercellular communications, including cell proliferation and inflammation (1–3).

Given these crucial functions for lipid molecules, the regulation of the enzymes that control their metabolism and therefore modulate their intracellular levels assumes a critical role. Altered control of these enzymes may result in serious problems in maintaining the appropriate homeostasis and balance between intracellular lipid signals, which regulate overlapping or opposing cellular functions such as cell proliferation and growth arrest or apoptosis. A growing body of evidences sets the general process of cell transformation in such a scenario, where the whole network of cellular proliferation, survival, and differentiation processes do not respond properly to the incoming stimuli, and lipid-mediated signaling may be involved in this event.

Among the signaling lipid molecules, DAG and, more recently, ceramide have been a target for a large number of studies with particular relevance to the regulation of cell growth/viability and transformation. Elevation of DAG levels has been observed in response to mitogenic stimuli such as growth factors, ras, and other oncogenic or transforming agents (4–9). The most studied pathway for DAG generation is through phosphoinositide breakdown, whereby the concomitant increase in DAG and intracellular Ca2+1, secondary to inositol trisphosphate generation, synergistically activate protein kinase C (10–12). Other main mechanisms of DAG production are mediated by the consecutive action of phosphatidylcholine (PC)-specific phospholipase D and phosphatidate phosphohydrolase and by PC hydrolysis through a PC-specific PLC (PC-PLC) (for reviews, see Refs. 13 and 14).

On the other hand, increased ceramide levels are associated with the induction of cellular growth arrest and apoptosis.

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1 The abbreviations used are: DAG, diacylglycerol; SM, sphingomyelin; SMS, SM synthase; PBS, phosphate-buffered saline; Cer, ceramide; GlCer, glucosyceramide; PC, phosphatidylcholine; PLC, phospholipase C; FBS, fetal bovine serum; MEM, minimum essential medium; bBSMase, bacterial SMase; BSA, bovine serum albumin; dBSA, delipidated BSA; SMase, sphingomyelinase.

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These increases in ceramide are seen in response to a variety of stimuli (tumor necrosis factor-α, interleukin-1, interferon γ, vitamin D₃, serum deprivation) and in several different cell lines (15–23). Intracellular regulation of ceramide levels is controlled by a complex system of metabolic reactions, including the action of different sphingomyelinases (SMases), ceramidas, ceramide kinase, glucosyltransferase, ceramide synthase, and possibly dihydroceramide desaturase (for reviews, see Refs. 24–26).

One of the most intriguing enzymes that regulate ceramide levels is the phosphatidylinositolceramide phosphocholine transferase (sphingomyelin synthase; SMS), which transfers the phosphocholine group from PC to ceramide generating sphingomyelin (SM) and DAG (27–33). This enzyme therefore has the important ability to directly regulate, in opposite directions, ceramide and DAG levels within the cells, potentially controlling opposite cellular processes such as cell proliferation, growth arrest, and apoptosis (34). Despite the great biological potential of SMS, very little is known about the enzyme. The largest effort has been spent on studying its intracellular localization (28–51), but its exact location and distribution remains to be elucidated. Moreover, few, if any, reports are available in the literature on the regulation of this enzyme.

In this study, we investigated the SMS activity in normal and SV40-transformed human lung fibroblasts (W138). We provide evidence of an enhanced SMS activity in SV40-transformed W138 compared with normal W138. In particular, in the SV40-W138, we show the presence of a SMS activity that utilizes ceramide produced in the plasma membrane; this activity is almost completely absent in normal WI38. We also discuss the fascinating possibility that SMS activity could account for some of the functions that have been proposed for PC-PLC, the fascinating possibility that SMS activity could account for.

**Experimental Procedures**

**Materials**—Dublcco’s modified Eagle’s medium, minimum essential medium (MEM), all MEM solutions, and trypsin-EDTA were from Life Technologies, Inc. Fetal bovine serum (FBS) was from Summit Technology. HCloline chloride (1 mC/ml extracted used), L-Diethylaminoethyl-3H-labeled phosphatidylcholine (50 Ci/mmol, 1 mC/ml), [3H]-2-ATP (10 mC/ml, 3000 Ci/mmol), and ENHANCE™ spray were from NEN Life Science Products. L-a-phosphatidylcholine, sphingomyelin, and 1-oleoyl-2-acetylglycerol were from Avanti Polar Lipids. C₂₀-sphingomyelin and b-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-HCl were from Matreya. Ceramide type III, bacterial sphingomyelinase (from Staphylococcus aureus), and fatty acid-free BSA were from Sigma chemical company. D609 was from Calbiochem. Silica Gel 60 thin-layer chromatography plates were from Whatman.

Scintillation mixture Safety Solve was from Research Products International. All solvents were of AR grade and were from Mallinkrodt.

**Cell Culture**—Both normal and SV40-transformed human lung fibroblasts (W138) were obtained from the NIA Aging Cell Repository. Normal human lung fibroblasts (W138) were routinely grown in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose, buffered with Hepes (pH 7.4). Cells were seeded at 5000 cells/plate in regular medium and at 10,000 cells/plate in 10% FBS-supplemented medium. Cells were used and supplemented with 10% FBS. W138 cells were used up to passage number 24–26. SV40-transformed fibroblasts (SV40 W138) were maintained in MEM supplemented with MEM solutions of vitamins, essential and nonessential amino acids, and 10% FBS. Cells were maintained at 5% CO₂ at 37 °C.

**Synthesis of [3H]d-Erythro-C₂₀-ceramide**—The compound was synthesized by acylation of (2S,3R)-sphingosine with [3H][CH₃]COO and purified by thin layer chromatography (TLC) (chloroform: methanol:2 N NH₄OH). The specific activity of the obtained [3H]d-erythro-C₂₀-ceramide was 8.7 × 10⁶ cpm/mg (10 μg stock solution in ethanol) by dilution with unlabeled 10 mM d-erythro-C₂₀-ceramide. (2S,3R)-b-Erythro-32P-sphingosine was prepared in stereo-protective synthesis from l-serine (52, 53).

**Metabolic Labeling of Cellular SM and PC Pool and Treatment of Cells with Bacterial Sphingomyelinase (bSMase)**—Cells were seeded at 2 × 10⁶ cells/plate in PBS, PBS, and chased for 2 h. After washing with PBS again, cells were treated with 100 milliunits/ml bSMase for 25 min (10 ml of medium). Then, cells were washed twice with PBS and fresh complete medium (10 ml) was added to continue the incubation for up to additional 24 h. In some experiments, 10–50 μg/ml (aqueous solution) were added to the medium. All manipulations were in 10% PBS-supplemented medium.

**Measurements of [3H]Choline-labeled SM and PC**—After removing the medium, cells were washed and scraped in PBS. The suspensions were centrifuged at 350 × g for 5 min at 4 °C, and the pellets were stored at −70 °C. On the day of the measurements, pellets were resuspended in distilled water and briefly sonicated to obtain an homogeneous lysate. Part of it was used for protein determination (54) and part for lipid extraction with 2.5 ml of chloroform:methanol (2:1) (55). Total lipid extract was subjected to mild base hydrolysis (56), and [3H]Phosphatidylcholine was quantitated by measuring the radioactivity in the aqueous phase. [3H]Sphingomyelin was determined by TLC analysis in chloroform:methanol:acetic acid:H₂O (50:30:8.5), followed by scavenging and counting the radioactivity by liquid scintillation.

**Mass Measurements of Cellular PC and SM**—Total lipids were extracted from the cells according to the Bligh and Dyer method (57) starting from the same amount of proteins. PC and SM were separated by TLC using chloroform:methanol:acetic acid:H₂O (50:30:8.5). Lipids were visualized by exposure of the TLC plate to iodine vapors, and PC and SM spots were identified by comparison with authentic standards. PC and SM were scraped and eluted from the silica gel by an extraction according to the Bligh and Dyer protocol (this procedure gave us 99% elution efficiency). PC and SM levels were determined by measuring the amount (nmol) of inorganic phosphate (P) present in the organic phase (58) and were normalized to total phosphate initially present.

**Ceramide and Diacylglycerol Measurements**—Cells were harvested in methanol, and lipids were extracted using the Bligh and Dyer method (57). One ml and 0.3 ml (in duplicates) of chloroform out of the organic phase were aliquoted separately, dried down, and used, respectively, for ceramide and phosphatidylcholine measurements (58). Ceramide levels were evaluated using the Escherichia coli diacylglycerol kinase assay (59, 60). Briefly, the lipids were incubated at room temperature for 30 min in the presence of β-octylglucoside/dioleoyl-phosphatidyl glycerol micelles, 2 mM dithiothreitol, 5 μg of proteins from the diacylglycerol kinase membranes, and 2 mM ATP (mixed with [γ-32P]ATP) in a final volume of 100 μl. After the lipids were extracted by the Bligh and Dyer method, the reaction products were separated by TLC in chloroform: methanol:acetic acid:H₂O (50:50:15:10:5), and the radioactivity associated with the phosphatidic acid and the ceramide-P was measured. Ceramide levels and diacylglycerol levels were quantitated by using external standards and were normalized to phosphatidylcholine.

**Incubation of W138 Fibroblasts with [3H]C₂₀-ceramide and Analysis of [3H]Labeled Lipids**—Cells were seeded at 5 × 10⁵ cells/plate in regular medium supplemented with 10% FBS. After 2 days, medium was changed and after additional 12 h the cells (about 1.5–10⁶ cells/plate) were incubated with 5 μM [3H]C₂₀-ceramide (specific activity: 20 μCi/μmol) for 24 h, and supplemented with 1% FBS. [3H]C₂₀-ceramide was delivered in ethanol solution (0.5% ethanol in the medium). At the time points indicated in the figures, medium was removed and cells were washed twice with PBS. After centrifugation at 350 g for 5 min, medium and PBS were both checked for radioactivity. Cells were scraped on ice into glass extraction tubes with 2 ml of ethanol (W138) or 3 ml of chloroform:methanol:acetic acid:H₂O (2:1:0.5) (SV40 W138) and 5 ml of chloroform (W138) were added. After washing with PBS again, cells were treated with 100 milliunits/ml bSMase for 25 min (10 ml of medium). Then, cells were washed twice with PBS and fresh complete medium (10 ml) was added to continue the incubation for up to additional 24 h. In some experiments, 10–50 μg/ml (aqueous solution) were added to the medium. All manipulations were in 10% PBS-supplemented medium.

**SM Synthase Assay**—W138 and SV40 W138 were collected after quick freezing. The cells were washed twice with PBS and homogenized in ice-cold lysis buffer by 10–15 passages through a 27-gauge × 0.5-inch needle. The lysis buffer contained 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml of each of leupeptin, antipain, and pepstatin. The cell lysates were first centrifuged at 1000 × g for 10 min at 4 °C in order to remove all the unbroken cells and nuclei. The postnuclear homogenates were then centrifugated at 100,000 × g for 1 h at 4 °C in order to get a whole cellular membrane preparation (pellet). Membrane proteins (150 μg) were preincubated for 10 min at 37 °C in a total final volume of 0.5 ml of incubation buffer containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl,
RESULTS

Uptake and Metabolism of $[^{3}H]C_{2}$-ceramide in Normal and SV40-transformed WI38 Cells—We treated the normal human lung fibroblasts, WI38, with 5 $\mu$M $[^{3}H]C_{2}$-ceramide in 1% FBS-supplemented medium for 24 h. Under these experimental conditions, the concentration of $C_{2}$-ceramide used did not induce cell death (trypan blue exclusion assay; data not shown), consistent with a previous study on the selective induction of senescence by ceramide in this cell line (61). This short-chain ceramide analog easily diffuses into the cells, overcoming permeability-related problems. We found that, after 15 min of incubation, over 50% of the total radioactivity was detected within the cells (Fig. 1A). The maximum level of radioactivity in the cells occurred after 5 h of incubation (about 70% of the total radioactivity), but decreased with time to 50% after 24 h of incubation.

During incubation, a significant portion of the $[^{3}H]C_{2}$-ceramide taken up by the cells was converted into two major products (Fig. 1B). After TLC analysis of the cellular lipids present in the organic phase after Bligh and Dyer extraction (57), the two compounds were present in addition to the $[^{3}H]C_{2}$-ceramide; the first one had an $R_{f}$ of 0.24 and identified as $[^{3}H]C_{2}$-SM by comparison with an authentic standard (Matreya), and the second compound had an $R_{f}$ of 0.52, and could be inhibited by D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol-HCl treatment (data not shown), and is therefore thought to belong to the ceramide family (most likely $C_{2}$-glucosyl ceramide) (43, 62). $[^{3}H]C_{2}$-ceramide metabolism was the most rapid during the first 2 h of incubation, and then it slowly down during the remaining incubation time (Fig. 1C). Concurrently, $C_{2}$-SM (Fig. 1E) and probably $C_{2}$-GICer (Fig. 1D) were produced at a maximum rate within the first 2 h of incubation. After this time $C_{2}$-SM was synthesized at a higher rate than the ceramide, and by 24 h of incubation $C_{2}$-SM and $C_{2}$-GICer accounted for about 13% and 9%, respectively, of the total radioactivity within the cell.

Interestingly, when comparing the effects of a similar treatment in SV40-transformed WI38 cells (SV40 WI38) with normal WI38 cells (WI38), a different situation was observed. First, whereas the maximum level of radioactivity detected inside the cell was the same (70% of the total radioactivity), it occurred within 2 h after labeling the SV40-transformed WI38 cells. Noticeably, the levels decreased more rapidly compared with the normal fibroblasts (40% decrease by 24 h of incubation, Fig. 1A). At the same time, the $[^{3}H]C_{2}$-ceramide that entered the cells was converted into the same products found for the normal WI38 fibroblasts. However, the metabolic rate was higher than that observed in the normal cell line. After 24 h of incubation, $[^{3}H]C_{2}$-ceramide accounted for only 53% of the total cellular radioactivity, whereas $C_{2}$-SM (Fig. 1E) and $C_{2}$-GICer (Fig. 1D) accounted for 24.5% and 22.5% of the total counts/min, respectively.

These results suggest the presence of SMS (Fig. 1F) and UDP-glucose:ceramide-glucosyl transferase (Fig. 1D) enzymatic activities in both normal and SV40-transformed WI38 cells. In the latter, the two activities appear significantly higher compared with the normal cell line. SMS activity has been reported in different cellular compartments such as cis/trans-Golgi and plasma membrane (28–31). Because the short chain ceramide analogs easily diffuse through the membranes, they can rapidly (probably minutes) reach different sites within the cell (43–45, 62). Therefore, it is likely that the SMS activity that occurs during in vivo $[^{3}H]C_{2}$-ceramide treatment (Fig. 1E) is a sum of the total enzymatic activity in the cell.

Treatment of Normal and SV40-transformed Fibroblasts with Bacterial Sphingomyelinase Reveals Presence of SMS—In order to specifically study the SMS activity associated with plasma membrane SM, normal and SV40 WI38 cells (1.5–2 × 10^6 cells/plate) were treated with bSMase (100 milliunits/ml) in 10% FBS-supplemented medium (Figs. 2 and 3). The presence of the bSMase in the medium induced hydrolysis of the SM located in the outer leaflet of the plasma membrane, with concomitant ceramide formation. After 25 min, the bSMase was removed with two washes in PBS, and fresh medium was added to the plates and incubated for an additional 24 h. As shown in Fig. 2, after 25 min of treatment with bSMase, massive SM hydrolysis was induced both in normal and transformed-WI38 cells as expected. In normal WI38 cells, 11% of SM (about 9.7 pmol of SM/nmol of P_i) could be recovered after bSMase treatment and 5.5–7% of SM (about 3 pmol of SM/nmol of P_i) in transformed WI38 (Figs. 2 and 3C). At the same time, the ceramide content dramatically increased (Fig. 2). In normal WI38 fibroblasts, it was elevated from 18 to 98 pmol/nmol of P_i (a change of about 80 pmol/nmol of P_i), whereas in the transformed WI38 it was elevated from 4.8 to 56.2 pmol/nmol of P_i (a change of about 50 pmol/nmol of P_i), corresponding to changes of approximately 5- and 11-fold, respectively. These changes in ceramide levels account for the amount of SM hydrolyzed in the two cell lines, respectively (Fig. 2).

Importantly, after generation of these very high levels of ceramide from SM hydrolysis, we observed two distinct responses of the two cell types in the further metabolism of ceramide and SM. Therefore, we investigated the ability of the two cell lines to clear the high levels of ceramide after washing out the bSMase. As shown in Table I, normal WI38 fibroblasts metabolized 2.2 pmol of ceramide/nmol of P_i and 19.2 pmol/nmol of P_i within 2 and 6 h, respectively, after washing out the bSMase. On the other hand, SV40-transformed WI38 cells were able to metabolize 26.1 pmol of ceramide/nmol of P_i and 41.1 pmol/nmol of P_i after 2 and 6 h, respectively, following washing away the bSMase enzyme. These results demonstrate a significant difference between normal and SV40-transformed WI38 in their ability to handle the ceramide produced after bSMase treatment. In the first 2 h, normal WI38 were almost unable to metabolize the produced ceramide compared with the efficient activity shown by the SV40 WI38, which were able to reduce the ceramide levels by half. Moreover, it is also important to note that bSMase treatment did not induce cell death or any major morphological changes appreciable by optical microscopy analysis, consistent with the inability of ceramide to induce death in this cell line, and consistent with the reported inability of exogenous bSMase to cause death in many cell types (63, 64). Thus, we proceeded to investigate the mechanisms that induced the different metabolic behavior.

We considered the possibility that SMS could be involved in
this phenomenon. For this, we measured the changes in the levels of SM after treatment with bSMase in both normal and SV40 WI38. Fig. 2 shows the SM measurements at the same time points as the changes in ceramide levels previously considered. Whereas the normal WI38 fibroblasts synthesized 0.7 pmol of SM/nmol of P₁ in 2 h and 1.7 pmol/nmol of P₁ in 6 h after washing out the bSMase, the SV40-transformed fibroblasts produced 26.2 pmol of SM/nmol of P₁ after 2 h and 40.6 pmol/
Sphingomyelin Synthase in Cell Transformation

After the 30-min time point, DAG levels steadily decreased, possibly because of the rapid metabolism of this molecule in the cell.

The changes in the levels of PC were barely detectable in the treated cells, with a progressive drop in labeling during the chase period seen in both cell lines (Fig. 3D). The lack of significant changes in PC levels is reasonable if we consider that the cellular concentration of this phospholipid is very high (Table III) (about 400 pmol/nmol of P₄ in both normal and SV40 W138) compared with SM (89 and 54 pmol/nmol of P₄, respectively, in normal and SV40 W138). Furthermore, PC turnover is more rapid than that observed for SM. In fact, about 60% of radioactive PC is lost within 24 h after [³H]choline labeling, whereas the loss of radioactivity associated to SM is hardly appreciable during this time (Fig. 3, C versus D, controls).

In Vitro Measurement of SM Synthase Activity in Normal and Transformed WI38 Cells—In order to further verify the differences in the SM synthase activity between normal and SV40-transformed cells observed after in vivo treatments, we measured in vitro basal SM synthase activity in the two cell lines. We used a modified protocol from Futerman and Pagano (36). [³H]C₂-ceramide was added as the labeled substrate for the reaction, as used for the in vivo treatment (Fig. 1). Under our experimental conditions, the enzymatic activity was linear with time and with the amount of membrane proteins (data not shown). Therefore, 150 µg of total membrane proteins, isolated both from normal or SV40-transformed W138 cells, were incubated with 20 nmol of [³H]C₂-ceramide (specific activity: ~1 × 10⁴ cpm/nmol) (Fig. 4). The reaction proceeded at 37 °C for 20, 30 and 60 min after 10 min of preincubation. The levels of [³H]C₂-SM produced by the transformed cell line were higher compared with those obtained from the normal W138 cells, for each time point examined and for all the membrane protein concentrations used (data not shown). After 20 min of incubation, normal W138 fibroblasts produced 26 pmol of SM/mg of protein and transformed-W138 cells produced 74 pmol/mg of protein. This difference in the synthase activity between the two cell lines is similar in magnitude to the results observed for the in vivo [³H]C₂-ceramide treatment (Fig. 1E). It is important to note that for the in vitro measurements we are actually monitoring a total membrane extract, which may contain different forms of SM synthase.

In Vitro Measurements of Basal SM Synthase Activity in Liver and Morris Hepatoma 3924A—Given the different SMS activity between normal and SV40 W138, we wanted to verify if any difference could be detected in other experimental models, comparing normal and transformed conditions. Therefore, we measured the basal SMS activity of normal liver and Morris hepatoma 3924A (a highly malignant tumor with a rapid growth rate) obtained from Aci/T rats. Liver and hepatoma samples were homogenized (10% w/v) in 0.25 M sucrose, 25 mM KCl, 50 mM Tris-HCl, 0.5 mM EDTA (pH 7.4) using a Potter-Elvehjem homogenizer. The homogenate was filtered through four layers of gauze and centrifuged at 1000 × g for 10 min. After a 10-min preincubation at 37 °C of the postnuclear homogenate (36), [³H]C₂-ceramide was added as the labeled substrate for the reaction, as used for the in vivo treatment. The SMS activity was measured as a function of [³H]C₂-SM production after 30 and 60 min of incubation, as described earlier. As shown in Table II, the homogenate obtained from the Morris hepatoma specimens showed a significantly higher SMS activity than the one detected in the homogenate prepared from normal liver. In fact, after 30 and 60 min of incubation, the hepatoma homogenates synthesized approximately 7 times more [³H]C₂-SM than normal liver homogenates.

Determination of Ceramide, DAG, SM, and PC Basal Levels

nmol of P₄, after 6 h (Table I and Fig. 2). This demonstrated that the W138 and SV40 W138 respond in a completely different way to the bSMase stimulus when the changes in SM levels were analyzed. In fact, very little SM production was observed in normal W138, whereas massive SM formation was detected in SV40 W138. Therefore, it could be concluded that normal W138 cells slowly metabolize the ceramide present in the plasma membrane, but it appears that very little if any of it is used for the resynthesis of SM. This indicated to us the possibility that normal W138 could be devoid of SMS activity able to utilize the specific pool of ceramide produced after bSMase treatment. SV40 W138 cells, on the other hand, were extremely active in reducing the levels of ceramide, almost completely converting it back to SM (after 6 h, 41.6 pmol of ceramide/nmol of P₄ cleared and 40.6 pmol of SM/nmol of P₄ produced).

Because the biochemical reaction catalyzed by the SMS also involves the consumption of PC and the production of DAG (27–31), we evaluated the levels of these compounds in response to the bSMase treatment (Fig. 3, B and D). The DAG level in the SV40 W138 increased moderately during the 25-min treatment with bSMase, and it continued to increase up to a peak at 30 min after washing out the bSMase (Fig. 2D, inset).

FIG. 2. Sphingomyelin synthase activity in response to bSMase treatment in normal and SV40-transformed W138: mass changes of ceramide and SM levels. Cells (1.5–2 × 10⁵ cells/plate) were incubated with 100 milliunits/ml bSMase for 25 min in 10% FBS-supplemented medium. After this time (indicated by the arrow on the x axis) cells were washed twice with PBS and were incubated with fresh normal medium for additional 24 h. Ceramide (cer) and SM levels were measured at the time indicated in the figures as described under “Experimental Procedures.” The values reported are representative of two or three independent experiments.
in Normal and SV40 WI38 Fibroblasts—SM synthase transfers the phosphorylcholine moiety from phosphatidylcholine to ceramide with the formation of SM and the production of DAG. Given the difference in the SMS activity we found in vivo and in vitro between normal and SV40-transformed WI38 cells, we wondered if this could affect, in the two cell lines, the basal cellular levels of ceramide, PC, SM, and DAG, the substrates and products of the reaction regulated by SMS. Therefore, the basal amount of the four lipids was measured in both normal and transformed WI38 fibroblasts (Table III). We found that DAG levels were approximately 40% higher in the transformed cell line than in the normal fibroblasts, whereas the SM levels were higher in the normal compared with the transformed cells. Because DAG and ceramide may have opposing biological functions (17, 65), it is of particular significance to evaluate the results as DAG/ceramide ratio. In this case, the data are even more striking; the DAG/ceramide ratio is over 5-fold higher in transformed than in normal cells.

The results are less clear when measuring the cellular levels of PC and SM. PC levels did not appear to be very different between the two cell lines, whereas the SM levels were higher in the normal than in the SV40-transformed cells. However, when considering the SM/ceramide ratio, it was still over 2-fold higher in the transformed than in the normal cells, consistent with the differences in SMS activity.

In Vitro and in Vivo Inhibition of SM Synthase Activity by the Putative “PC-PLC-specific inhibitor” D609—From the data shown, it became evident that SM synthase activity was elevated in SV40-transformed WI38 cells compared with the normal counterpart. It has been reported that the activity of a PC-PLC may play an important role in the process of cellular transformation (4, 13, 66–70). Because this enzyme is thought to cleave PC with the production of DAG and choline-P, we investigated the possibility that PC-PLC activity could have been also relevant in inducing the differences we found between the two WI38 cell lines. For this, we evaluated any [3H]choline-P production during in vitro incubation of total membrane extract (150 μg of proteins), isolated from SV40-transformed WI38 cells, in the presence of [3H-choline]phosphatidylcholine (Table IV). After 1 h of incubation at 37 °C, in the presence of either 50 or 100 nmol of labeled PC, no production of labeled choline-P was detected. Instead, when checking for production of labeled SM under the same experimental conditions, we found significant activity with both amounts of labeled PC used (160 and 331 cpm of generated SM with 50 and 100 nmol of PC, respectively). Thus, we could not detect any PC-PLC activity in the SV40-transformed WI38.

When comparing substrates and products of the reactions catalyzed by SM synthase and PC-PLC, it is interesting to note...
that for both reactions PC is used as substrate and DAG is one of the products (see Fig. 6). Besides, both enzymes may be localized to the plasma membrane and both of them appear to be differently regulated in normal and transformed cells. Moreover, there has been little documentation of genuine PC-PLC activity at a biochemical level in mammalian cells. Because of the intriguing similarities between PC-PLC and SMS, we wondered whether many (or at least some) of the reported effects and regulators of PC-PLC might not be more directly attributable to SMS. It seemed to us particularly interesting to study the effect of the purported PC-PLC-specific inhibitor, D609 (71, 72), on SM synthase activity. For this, we incubated different concentrations of D609 (from 50 up to 200 \( \mu \)g/ml) with a total membrane extract (150 \( \mu \)g of proteins) isolated from SV40-transformed WI38 fibroblasts (Fig. 5A). Before the addition of 20 nmol of \(^{3}H\)C\(_2\)-ceramide in ethanol solution (0.2% EtOH in the incubation mixture), we preincubated the membrane suspension for 20 min at 37 °C. Then, we measured the \(^{3}H\)C\(_2\)-SM production after 60 min of incubation. The addition of D609 induced inhibition (about 30%) of SM synthase activity at the lowest concentration used (50 \( \mu \)g/ml). Increasing D609 concentrations caused increasing levels of inhibition of SM synthase: 55.5% and 90.5% inhibition of \(^{3}H\)C\(_2\)-SM production, respectively.
tively, with 100 μg/ml and 200 μg/ml D609 (Fig. 5A). Because D609 inhibited SM synthase activity in vitro, we wanted to verify the effect of an in vivo treatment in SV40-transformed WI38 cells using concentrations of D609 claimed to be specific for inhibition of PC-PLC (Fig. 5B). For this, we treated the cells with bSMase (100 milliunits/ml) for 25 min as shown previously (Fig. 2), in order to monitor maximum SM synthase activity. After washing out the bSMase, 25 μg/ml D609 was added to the medium, and cell samples were collected after 2, 4, and 6 h of incubation. SM levels were evaluated; addition of D609 totally inhibited SM production after 2 h of incubation, and it was still inhibitory by 75–80% after 6 h. Very similar results were obtained when concentrations of 10 and 50 μg/ml D609 were similarly tested on SMS activity (data not shown).

**DISCUSSION**

In the present study, SMS activity was investigated in normal and SV40-transformed human lung fibroblasts (WI38). Our results show that SV40-transformed WI38 cells contain a significantly higher SMS activity than normal WI38. Our results further suggest the existence of a specific SMS activity that acts on ceramide generated in the plasma membrane. This activity is almost absent in wild type cells but highly enriched in transformed cells. The properties of this enzyme raise the tantalizing possibility that it could account for some or many of the proposed functions of the purported, but elusive, mammalian PC-PLC.

Evidence for the increased activity of SMS in SV40 WI38 was provided by multiple lines of evidence. First, SMS activity was investigated in vivo as the ability of the cells to metabolize radiolabeled C2-Cer producing labeled C2-SM. In this case, SV40-transformed WI38 were able to produce twice the amount of C2-SM as the normal WI38. Second, very similar results were obtained when SMS activity was assayed in vitro. Whole membrane preparations isolated from cellular lysates of WI38 and SV40 WI38 were incubated with C2-Cer, and C2-SM production was followed. The SMS activity present in the SV40 WI38 membrane fraction was double the activity monitored in the normal WI38 membrane fraction. Third, and in agreement with the presence of an enhanced SMS activity, cellular ceramide and DAG levels measured in SV40 WI38 were lower and higher, respectively, compared with the levels of the same lipids present in normal WI38. These results clearly show that SMS activity is present in both cell lines, and that it is 2-fold higher in the SV40-transformed WI38 than in normal WI38.

Interestingly, when elevation of a specific pool of cellular ceramide was induced in both fibroblasts by treatment with bSMase, a dramatically different SMS activity profile was observed in the two cell lines, and the difference between the two cell lines became almost absolute. Thus, WI38 were hardly able to metabolize the ceramide produced in the plasma membrane by the bSMase treatment, and they were almost completely unable to produce SM from it. On the other hand, SV40 WI38 actively metabolized this ceramide converting it back to SM.

The results from the treatment of normal and SV40 WI38 with bSMase led us to consider the interesting possibility that the main difference in SMS activity between these two cell lines is perhaps due to a form of the enzyme that resides in the plasma membrane or in functional proximity to it (enzyme that can act on ceramide generated in the plasma membrane). On the other hand, the treatment with C2-Cer does not allow us to discern between SM synthesis occurring in different cellular compartments, inasmuch as C2-Cer, like other short chain ceramide analogs, is readily taken up by cells and probably diffuses very quickly reaching different sites within the cells (43–45, 62). Because of this, C2-SM production would be a measure of total SMS activity present in the cells. In this case, the difference between normal and transformed WI38 is not as dramatic as it is after bSMase treatment. Similarly, there were more moderate differences when SMS activity was assayed in vivo using the whole cellular membrane fraction as source of the enzyme, because this procedure includes total SMS activity. Based on these and other results, one may hypothesize the presence of at least two SMSs, one more directly involved in the de novo synthesis and the second SMS more connected preferentially to plasma membrane ceramide. Indeed, the data available in the literature from subcellular fractionation studies seem to localize most of the SMS activity in the cis medial Golgi (36, 37, 41, 43–45, 47) with a component of the activity in the plasma membrane (28, 30, 31, 36, 46, 50, 51), endoplasmic reticulum (29, 49), and mitochondria (48), whereas it seems absent in the endosomes (73). Additionally, it has been proposed that the SMS activities present in different cellular compartments may be involved in different biological processes. In particular, it has been already suggested that the SMS localized in the Golgi could be more specifically responsible for the de novo synthesis of SM, whereas the form localized in the plasma membrane could play a more specific role in signal transduction events (58, 74).

The difference in the SMS activity between normal and SV40-transformed WI38 raises the important possibility of a role for SMS in the regulation of cell growth and function through the modulation of the levels of ceramide and DAG. Through its reaction (Fig. 6), the enzyme has the ability to regulate, in opposite directions, the intracellular levels of ceramide and DAG. Both of these molecules have been shown to be important bioeffector or second messenger lipids. Ceramide has been shown to mediate and mimic the ability of some effectors (such as tumor necrosis factor-α, vitamin D3, interleukin-1) to induce apoptosis, growth arrest and other metabolic and signaling functions (15–26). DAG, on the other hand, has been shown to play an antiapoptotic and stimulatory role for cell proliferation in addition to other signaling functions (for reviews, see Refs. 13 and 14). Thus, it appears that these two lipids exert different, if not opposite, roles in controlling the processes that lead cells to undergo cell death or growth arrest or to progress in the cell cycle (65). It seems to us that the regulation of the ratio between intracellular ceramide and DAG levels may be an important “biostat” in the regulation of cell viability/cell death and possibly other functions. If that is the case, SMS may be a component in the direct regulation of this balance. In this perspective, the 5-fold increase of the DAG/ceramide levels ratio in SV40-transformed WI38 with respect to normal WI38 and the complete absence of the plasma membrane-related SMS activity in the latter provide a promising starting point for further investigation in this direction.

It should also be noted that the difference in SMS activity between the two cell lines, while reversing the ratio of DAG/Cer, was not associated with a major difference in PC or SM levels. We could hypothesize that the SMS in endoplasmic reticulum/Golgi is the primary determinant of steady state levels of SM through regulation of de novo synthesis whereas the SMS directed at ceramide generated in the plasma membrane may be a primary regulator of the DAG/ceramide ratio.

The unexpected inhibition of SMS activity by the specific PC-PLC inhibitor D609 makes the whole argument even more intriguing. PC-PLC and SMS share several biochemical, topological and biological characteristics. PC-PLC hydrolyzes PC with subsequent DAG and choline-P production, even if genuine biochemical studies are missing; on the other hand, SMS is also able to convert PC into DAG (Fig. 6). Both enzymes seem to function at neutral pH, and both appear to localize to the plasma membrane. Moreover, elevated activity of both en-
enzymes seems to be associated with the transformed phenotype. For example, enhanced PC-PLC activity was observed in ras- or src-transformed cell lines (4, 66–70, 72), whereas SMS enhanced activity (as evaluated by changes in PC and DAG) is correlated in this case to SV40 transformation and to other neoplastic conditions such as hepatomas. Although, it has been reported by several authors that both the molar percentage of SM and the SM/PC ratio are increased in Morris hepatomas (74–78). In our hands, in vitro measurements show a dramatic increase (about 7-fold) of SMS activity in homogenates prepared from Morris hepatomas 3924A compared with those prepared from normal livers (Table II). These results support a previous report on SMS activity in hepatomas (79). In that study, utilizing subfractionation procedures, SMS activity was found predominantly in the plasma membrane and it was elevated (1.5–3.0-fold) compared with host liver.

All the similarities between PC-PLC and SMS and, most importantly, the susceptibility of SMS activity to the specific PC-PLC inhibitor, D609, led us to consider the possibility that an increased SMS activity, possibly due to a plasma-membrane related form of the enzyme, is associated with SV40-transformed phenotype in human lung WI38 fibroblasts and in other neoplastic conditions. This activity shows many similarities with PC-PLC and it is efficiently inhibited by the PC-PLC inhibitor, D609. These data raise the question whether the biological events that until now have been attributed to the PC-PLC may be due partially to the SMS. More importantly, these results support a role for SMS in cell regulation and transformation through its ability to modulate the levels of ceramide and DAG.

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![Figure 6](image-url)
Sphingomyelin Synthase in Cell Transformation

14559

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