Identification of a Distinct Pool of Sphingomyelin Involved in the Sphingomyelin Cycle*

(Received for publication, April 8, 1994, and in revised form, June 29, 1994)

Corinne M. Linardic and Yusuf A. Hannun†

From the Departments of Medicine and Cell Biology, Duke University, Durham, North Carolina 27710

Sphingomyelin (SM) is a membrane phosphosphingolipid that has recently been identified as a key component of the SM cycle. In this signal transduction pathway, extracellular inducers such as tumor necrosis factor α cause hydrolysis of membrane SM, resulting in the generation of the lipid second messenger ceramide. Only 10–20% of cellular SM appears to be involved in the SM cycle, raising the possibility of the existence of a unique “signaling” pool of SM. The existence and subcellular location of such a pool was investigated. Using bacterial sphingomyelinase from Staphylococcus aureus (bSMase), we first characterized two pools of SM, identified as an outer leaflet bSMase-sensitive pool and a distinct bSMase-resistant pool. These pools were further characterized by their differential solubility in Triton X-100 and by their kinetics of labeling. The signaling pool of SM was distinguished by the following: 1) resistance to bSMase, 2) solubility in Triton X-100, and 3) delayed labeling kinetics. In subfractionation studies, the signaling pool of SM co-fractionated with the plasma membrane. Since the SM cycle involves a cytosolic sphingomyelinase and the intracellular release of choline phosphate, this pool of SM appears to localize to the inner leaflet of the plasma membrane (or to a closely related compartment). These results identify a unique signaling pool of SM that undergoes significant hydrolysis (20–40%) in response to inducers of the SM cycle.

Sphingomyelin (SM)† is a membrane phosphosphingolipid that functions in the regulation of membrane fluidity (1, 2) and participates in discrete microdomains of the outer leaflet of the plasma membrane (Ref. 3 and references cited therein). Recent studies have also identified a critical role for SM in the regulation of membrane fluidity (1, 2) and participate in discrete microdomains of the outer leaflet of the plasma membrane. Additionally, studies on the biosynthesis of SM showed that it was synthesized in the cis and medial Golgi apparatus, suggesting yet another intracellular compartment containing SM (38).

On the other hand, other studies on the topology of SM suggested that it might exist in other compartments. A number of studies on erythrocytes (29, 30), nucleated mammalian cells (24, 25), and the lipid coats of budding viruses (which reflect the plasma membrane of the host cell) (31–37), suggested that SM might also exist within the inner leaflet of the plasma membrane. Additionally, studies on the biosynthesis of SM showed that it was synthesized in the cis and medial Golgi apparatus, suggesting yet another intracellular compartment containing SM (38).

In the course of exploring the SM cycle of cell regulation, two observations emerged that contradict a simple model whereby
SM is localized exclusively to the outer leaflet of the plasma membrane. First, the choline phosphate breakdown product of SM was observed to increase in the cell pellet but not in the medium during activation of the SM cycle (6). This was unexpected, since choline phosphate generated from the breakdown of SM of the outer leaflet pool would be released into the surrounding medium. Second, the SMase enzyme activated in the SM cycle was isolated from the cytosol (6, 39), again suggesting an intracellular localization of the signaling pool of SM.

In the present study we provide evidence for the existence of at least two distinct pools of SM. A first pool of SM was readily identified as belonging to the outer leaflet of the plasma membrane, determined by susceptibility to the action of extracellular SMase and co-fractionation with the plasma membrane. A second distinct signaling pool of SM was characterized by resistance to the action of extracellular SMase. It is also distinguished as belonging to a Triton X-100-soluble pool of membrane sphingolipids. Cell fractionation studies show that the signaling pool fractionates with the plasma membrane fraction. The signaling pool of SM also shows distinct metabolic labeling properties from the outer leaflet pool. Taken together, these studies define a unique signaling pool of SM. The implications of these results are discussed.

EXPERIMENTAL PROCEDURES

Materials—All media, media supplements, and fetal calf serum were from Life Technologies, Inc. [3H]UDP-glucosamine and [3H]choline chloride were from DuPont NEN. TNFα was a generous gift from Knoll Pharmaceuticals, Whippney, NJ, and 1,2,5-dihydroxystigmine D3 was a generous gift from Dr. Milan Uskokovic, Hoffmann-La Roche, Nutley, NJ. Archidonic acid was from Matraya, Inc., Pleasant Gap, PA. Percoll and density gradient beads were from Pharmacia Biotech Inc. All other reagents were from Sigma.

Cell Culture—HL-60 and U937 human leukemia cells were purchased from the ATCC, Rockville, MD and maintained in RPMI 1640 media supplemented with 10% fetal calf serum. Cells were maintained at densities between 2 × 10⁴ to 5 × 10⁵ cells/ml under standard incubator conditions (humidified atmosphere, 95% air, 5% CO₂, 37°C).

Metabolic Labeling of Cellular SM Pools—In order to label SM pools, cells were incubated with [3H]choline for 48-72 h, as described previously (13). Briefly, cells from maintenance culture were washed once in phosphate-buffered saline and resuspended to 5 × 10⁵ cells/ml in serum-free RPMI 1640 (serum-free media) supplemented with insulin/transferrin/sodium selenite as serum replacement. [3H]choline was added to a final specific activity of 0.50 μCi/ml. After labeling, radiolabeled SM was extracted with phosphate-buffered saline, and cells were resuspended in serum-free media to a density appropriate for the experiment. Before further experimental manipulation, cells were preincubated for 2-3 h under standard incubator conditions as described under "Cell Culture." Measurement of SM and Phosphatidyethanolamine (PC)—SM and PC were quantitated as described in Ref. 13. However, some experiments were done using a recently published rapid assay for SM and PC (12). This assay relies on the use of bacterial sphingomyelinase from Streptomyces sp. to specifically hydrolyze [3H]choline-labeled SM (100% hydrolysis) with no detectable hydrolysis of PC. Controls demonstrate that results from this rapid assay are similar to those results derived from traditional quantitation by thin layer chromatography, whether utilizing exogenous substrate or membrane lipids. This modified assay has been described in detail in Ref. 12, but it will be outlined here. Briefly, after Bligh-Dyer (40) extraction, a portion of the lower (organic) phase of the cellular lipid extraction was dried under N₂ gas and resuspended as a mixed micelle in 0.10% Triton X-100 buffered with 0.1 M Tris-HCl, pH 7.5, and 6 mM MgCl₂. After vigorous vortexing and short bursts of sonication to disperse lipids into micelles, samples were preincubated for 15 min at 37°C and then treated for 2 h at 37°C with 1 μl/ml of bacterial sphingomyelinase from Streptomyces. Each sample was then extracted using the method of Folch and co-workers (41). Reactions (100 μl) were stopped by the addition of 1.5 ml of chloroform/methanol (2:1). The monophosphate was then broken with 200 μl of water. The fluid was vortexed and centrifuged to a biphasic, and 400 μl was aspirated from the upper-phase containing PC and transferred to a second tube. The upper phase represented the choline phosphate generated from SM hydrolysis. A volume of 600 μl was then collected from the lower phase, dried to remove traces of chloroform (which has quenching effects) and counted. Since all SM was hydrolyzed by the bacterial enzyme, counts/min from the lower phase represent only PC. As with the previously used TLC separation method, SM counts/min were normalized by PC counts/min or by phospholipid phosphorus measurements.

Treatment of Cells with Bacterial Sphingomyelinase—Cells were resuspended in 1.5 × 10⁶ cells/ml in serum-free medium buffered with 50 mM HEPES pH 7.4 and preincubated for 15 min at 37°C. Bacterial sphingomyelinase from Staphylococcus aureus was added at a final concentration of 100 milliunits/ml for 10 min at 37°C. This concentration is sufficient to cleave outer leaflet SM (see Fig. 1). Control cells were treated with the same volume of culture medium (50% glycerol in 0.25 M phosphate buffer, pH 7.4). If cells were to be used in further manipulations, such as Triton X-100 fractionation or Percoll fractionation (see below), they were washed in phosphate-buffered saline containing 10 mM EDTA in order to inhibit the S. aureus sphingomyelinase, which is known to be Mg²⁺-dependent (42). In vitro studies utilizing mixed micelles of 10% Triton X-100/10 mM of [3H]phosphatidylcholine from HL-60 cells reconstituted in EDTA or Mg²⁺-containing Tris buffer, pH 7.5, as substrate, demonstrate that simple chelation of 1 mM MgCl₂ with 10 mM EDTA inhibits S. aureus sphingomyelinase activity completely.

Fractionation of Cellular SM Using Triton X-100 Insolubility—Cellular SM was fractionated based on solubility in Triton X-100 at 4°C as described for sphingolipids by Brown and Rose (3). Briefly, approximately 8 × 10⁶ cells metabolically labeled with [3H]choline were washed once, resuspended and lysed in 1 ml of ice-cold 1% Triton X-100 extraction buffer (containing 25 mM HEPES pH 7.5, 0.15 M NaCl, and 100 μM aminooxyacetic acid), and then incubated for 20 min on ice. This should be sufficient to inhibit S. aureus sphingomyelinase, 10 mM EDTA was included in the extraction buffer in order to chelate Mg²⁺ and inhibit the remaining trace amounts of this enzyme. Triton X-100-insoluble material was pelleted at 12,500 × g in a Microfuge at 4°C, and the detergent-soluble material was collected and transferred to a new tube. The Triton X-100-insoluble pellet was reconstituted, and the residual soluble material was again collected and combined with the first harvest of soluble material. Both fractions were analyzed for SM and PC content. The entire Triton X-100-insoluble pellet was dissolved in 1 ml of chloroform/methanol (1:2) and extracted (by the method of Bligh and Dyer) while 0.80 ml of the soluble material was treated with equal volumes of vehicle (50% glycerol in 0.25 M phosphate buffer, pH 7.4). Using niton X-ray sulfur radiation, because we have determined that disruption of cells by detergent causes a loss in cellular SM, probably by activation of an endogenous sphingomyelase, we included whole cell (unfractionated) controls in our experiments in order to quantitate percent SM lost due to the Triton X-100 disruption procedure.

Fractionation of Cells into Nuclear and Extranuclear Fractions—The method of Bunce et al. (43) was used to separate HL-60 into nuclear and extranuclear fractions. Briefly, cells were metabolically labeled with [3H]choline were washed three times with 5 ml of ice-cold nuclear prep buffer (NBP; 10 mM Tris-HCl, pH 7.4, 0.14 M NaCl, 2 mM MgCl₂) and then resuspended in 1 ml of ice-cold NBP with 2% (v/v) Triton X-100 detergent in a Microfuge tube. The tube was immersed in liquid N₂ and then thawed under running hot water. The freeze-thawed cells were then transferred into a precooled glass Dounce chamber and homogenized with 20 strokes of a Teflon pestle. The resulting homogenate was layered over a 250-ml cushion of 50% (w/v) sucrose in NPB and centrifuged in a Microfuge for 1 min at 13,500 rpm. Nuclear pellet harvested at the bottom of the sucrose cushion, while the extranuclear fraction was retained above the sucrose cushion. The extranuclear fraction was harvested by aspiration, while the sucrose cushion (being careful to avoid the nuclear pellet) was discarded. The remaining nuclear pellet was then harvested by slicing off the bottom tip of the Microfuge tube. Both fractions were analyzed for [3H]choline-labeled SM and counts/min were normalized to phosphatidylphosphate as for the whole cell studies as described above.

Fractionation of Membranes Using Percoll Gradients—In our search for rapid methods of membrane fractionation for myeloid cells, we adopted the method of Record et al. (44), which was developed for the fractionation of rat liver and mouse lung. In this procedure, the cellular homogenate is raised to an alkaline pH (9.2-9.6) in order to facilitate separation of the plasma membrane and intracellular organelar membranes on Percoll gradients. After pilot experiments, we determined that membranes from HL-60 cells were not separating as predicted. Therefore, we introduced some changes in the original protocol. In the first change, the starting Percoll density (1.065 g/ml) was raised to a continuous gradient rather than a step gradient, and changes in centrifugation
RESULTS

Identification and Characterization of Two Distinct Pools of SM—Our first goal was to determine if SM existed in distinct pools. In an initial approach we treated HL-60 and U937 cells with bSMase from S. aureus to hydrolyze outer leaflet SM. In both cell types, approximately 60% of the total cellular SM was hydrolyzed by this treatment (Fig. 1), corresponding to an outer leaflet pool. The remaining 40% was not hydrolyzed by S. aureus SMase. Importantly, the two pools are not in rapid equilibrium since prolonged (Fig. 1) or repeated (data not shown) application of bSMase does not result in hydrolysis of the resistant pool. Thus, the action of bacterial SMase identified two distinct pools of SM, bSMase-sensitive and bSMase-resistant.

We next evaluated additional methods to distinguish the two pools of SM. Recent studies have shown that sphingolipids (including SM) and glycosphingolipid-inositol-anchored proteins associate together in an insoluble cluster when whole cells are disrupted by Triton X-100 at 4 °C (3). Thus, we hypothesized that we might be able to distinguish the bSMase-sensitive and -resistant pools of SM based on their Triton X-100 solubility. We used metabolically labeled HL-60 and U937 cells with bSMase-resistant pool and not from a decrease in the bSMase-sensitive pool. From these isotope incorporation studies, we conclude that the incorporation of choline into specific SM pools varies
A Distinct Signaling Pool of Sphingomyelin

FIG. 2. Use of Triton X-100 4 °C fractionation to identify detergent-soluble and detergent-insoluble SM pools. HL-60 cells labeled with [3H]choline for 48–72 h were lysed with 1% Triton X-100 in HEPES-NaCl buffer at 4 °C as described under “Experimental Procedures.” Repeated centrifugation in a Microfuge separated the Triton X-100-soluble material from the Triton X-100-insoluble material. Both fractions were analyzed for SM and PC content. A, fractionation of cells in Triton X-100 results in the separation of total SM counts/min into a Triton X-100-soluble pool and a Triton X-100-insoluble pool. B, pretreatment of cells with bSMase prior to fractionation in Triton X-100 reveals that the insoluble pool is largely bSMase-sensitive. In this analysis, SM counts/min were normalized to PC counts/min, mean ± S.D.

with the duration of labeling and possibly other factors such as growth rate.

The SM Signaling Pool Is Resistant to bSMase—Our next goal after defining and characterizing the intracellular and outer leaflet pools of SM in control cells was to investigate the role of these pools in the SM cycle and, thus, in signal transduction. Our primary question was whether the signaling pool was located within the bSMase-sensitive or the bSMase-resistant SM pools. To address this question, metabolically labeled cells were treated with bSMase to define the two pools (as in previous experiments) and with inducers of the SM cycle. If the signaling pool resides in the outer leaflet of the cell then treatment with bSMase would eliminate the signal seen with inducer. If the signaling pool was present in a different compartment (e.g., intracellularly) then those cells treated with inducer and bSMase would show a further loss in SM in addition to the loss caused by bSMase. HL-60 cells were treated with known inducers of the SM cycle, either 100 nM 1,25-dihydroxyvitamin D₃ (6) or 30 nM TNFα (7). Parallel samples were treated with inducer alone or with bSMase.

Treatment with inducer (1,25-dihydroxyvitamin D₃ or TNFα) plus bSMase resulted in greater SM loss than with bSMase alone (Fig. 4). As shown in Fig. 4A, treatment with 100 nM 1,25-dihydroxyvitamin D₃ alone resulted in a loss of 15.4% of total SM, thus defining the 1,25-dihydroxyvitamin D₃-responsive pool. Treatment with bSMase resulted in a loss of 48% of total cellular SM, thus defining the outer leaflet pool. Treatment with both 1,25-dihydroxyvitamin D₃ and bSMase resulted in a loss of 65.3% of total SM. Since the loss in SM due to 1,25-dihydroxyvitamin D₃ is preserved during bSMase treatment, the signaling pool belongs to the bSMase-resistant pool of SM. Shown in Fig. 4B is a similar study using TNFα as an inducer of the SM cycle. Treatment with 30 nM TNFα alone resulted in a loss of 7.7% of total SM, while treatment with bSMase resulted in a loss of 44.6% of total cellular SM. Treatment with both TNFα and bSMase resulted in a loss of 51.9% of total SM. We, therefore, conclude that the sphingomyelin signaling pool is bSMase-resistant and that the two inducers appear to cause hydrolysis of the same pool of SM.

Membrane Fractionation Studies—Since the signaling pool of SM is distinct from the plasma membrane outer leaflet bSMase-sensitive pool, studies were conducted to identify the subcellular membrane(s) involved in SM cycle signaling. Initially, nuclei and extranuclear membranes were separated and examined for the signaling pool. HL-60 cells were metabolically labeled with [3H]choline, treated with 100 nM 1,25-dihydroxyvitamin D₃ for 3.5 h, and then fractionated into nuclear and extranuclear fractions. The signaling pool was present in the extranuclear fraction but not in the nuclear fraction (data not shown). In addition, we determined that the total mass of SM residing in the nucleus corresponded to less than 2% of total cellular SM. From these data, we could eliminate the nucleus as the site of SM breakdown and instead focus on the extranuclear membranes.
A Distinct Signaling Pool of Sphingomyelin

Fig. 4. Treatment of HL-60 cells with SM cycle inducers and bSMase. HL-60 cells metabolically labeled with $^{3}$Hcholine were washed free of radioactivity and resuspended to $5 \times 10^5$ cells/ml. They were then treated with an inducer of SM turnover, 1,25-dihydroxyvitamin D$_3$, or the appropriate vehicle, bSMase, or inducer plus bSMase. A, 100 nM 1,25-dihydroxyvitamin D$_3$ treatment for 2.5 h; B, 30 nM TNF+$\alpha$ treatment for 50 min. Results are expressed as percent of control, mean ± S.D. for A and mean ± range for B.

We next utilized fractionation on self-generating Percoll gradients to separate and examine extranuclear membranes. HL-60 or U937 cells (1–2 $\times 10^6$) were homogenized by nitrogen cavitation, and 5 ml of postnuclear supernatant was collected and separated on a Percoll buffer mixture in an ultracentrifuge in order to rapidly generate gradients in situ. Fractions were collected and analyzed for phospholipid phosphate content. Membranes were concentrated in two peaks (Fig. 5A). Analysis of the $^{3}$Hcholine-labeled SM content of these membranes also showed two major peaks of SM (Fig. 5B). In order to determine the membrane content of these peaks, organelle membrane markers were assayed in the fractions: as shown in Fig. 5C, peak 1 was enriched in endoplasmic reticulum (sulfatase C), while peak 2 was enriched in plasma membrane (alkaline phosphatase). Lysosomal membranes (acid sphingomyelinase) were also found enriched in peak 1. Further studies using this method of fractionation were simplified by the harvesting of three major fractions, with fraction 1 containing membrane from peak 1 (enriched in endoplasmic reticulum and lysosomes), fraction 2 containing membrane from the middle of the gradient (which was enriched in galactosyltransferase, a marker of Golgi), and fraction 3 containing membrane from
peak 3 (enriched in plasma membrane). Fractionation on Percoll was then used to analyze the membranes of both HL-60 and U937 cells. Since we were interested in defining the intracellular versus the outer leaflet pools of SM, we fractionated cells that had previously been treated with bSMase to remove outer leaflet SM. Most of the SM loss due to the bacterial SMase occurred in fraction 3 of the gradient, corresponding to the plasma membrane fraction; some amount of SM was also lost in fraction 2, indicating some contamination of fraction 2 with plasma membrane (Fig. 6). A significant amount of SM persisted in the plasma membrane fraction (fraction 3) following bSMase; this indicated the presence of a resistant pool of SM in the plasma membrane fraction.

As we had done for the whole cell analysis, we investigated the kinetics of labeling of HL-60 membrane SM by fractionation on Percoll gradients. We labeled cells for 24 or 72 h with [3H]choline, homogenized them, and then fractionated the post-nuclear supernatants on Percoll. With increasing time, the distribution of [3H]choline-labeled SM changed. Early in the duration of labeling, at 24 h, fraction 2 was enriched in [3H]SM (Fig. 7A). Since the Golgi membranes are enriched in this fraction, we suggest that this represents synthesis of [3H]SM from [3H]choline-labeled phosphatidylcholine. At 72 h, fraction 3 was greatly enriched in [3H]SM (Fig. 7B), while [3H]SM was lost from the first 2 fractions, suggesting enhanced labeling of the plasma membrane SM. We conclude that at different durations of labeling, different pools of SM are labeled. Most notably, the plasma membrane fraction, fraction 3, is significantly labeled later than the other membranes. These data correspond with the kinetic labeling studies done in whole cells, which show that an extra pool of SM is labeled very late in the course of metabolic labeling.

We next used the Percoll gradients in order to localize the signaling pool to a specific membrane fraction. To this end, cells were labeled with [3H]choline and then split into two groups. One group was treated with an inducer of the SM cycle, and the other was treated with vehicle. After the optimal incubation time, each group was fractionated on a Percoll gradient. As shown in Fig. 8, U937 cells treated with 30 nm TNFa for 10 min showed a loss of SM in peaks 2 and 3, corresponding to a total

DISCUSSION

The goal of these studies was to examine cellular SM pools in terms of their location and distribution in light of the recently described role of SM in signal transduction. In particular, an exclusive localization of SM to the outer leaflet of the plasma membrane defied a simple explanation for a mechanism of SM turnover, considering the cytosolic localization of hormone-activated neutral sphingomyelinase (39) and the intracellular release of the choline phosphate headgroup (6). Results from the present study demonstrate the existence of at least two separate and unique pools of SM as defined by the following: 1) sensitivity to bSMase, 2) solubilization by Triton X-100, 3) labeling kinetics, 4) fractionation on Percoll gradients, and 5) involvement in SM-mediated signal transduction.
The existence of multiple and unique pools of SM has only recently been considered. During our investigations of the SM signaling pool, other investigators noted the existence of two separate pools of SM as defined by bSMase. In their studies, performed in mouse Leydig tumor cells and baby hamster kidney (BHK21) cells, intracellular SM represented 30–50% of the total cell SM (51, 52). Interestingly, these values correlate very well with the values reported here, where intracellular SM ranges from 40 to 60%. The older studies described in the literature, which implicate SM as an outer leaflet lipid, were performed in erythrocytes and were based on, among other methods, susceptibility to bSMase. In this context, the use of the erythrocyte as a model cell in which to study plasma membrane SM topology (while superior in that erythrocytes contain methods, susceptibility to bSMase. In this context, the use of the erythrocyte as a model cell in which to study plasma membrane SM topology (while superior in that erythrocytes contain very little intracellular membrane to complicate analysis) could not address the distribution of intracellular SM. Additionally, the total sensitivity of erythrocytes to bSMase may be a property specific to erythrocytes, indicating the absence of inner leaflet SM in those cells. The only other studies demonstrating exclusive outer leaflet localization of SM are based on the use of fluorescent analogs of ceramide. The SM derived from these shorter chain NBD derivatives of ceramide may reflect one pathway of SM synthesis, resulting in outer leaflet localization, possibly because of the initial localization of NBD-ceramide to the Golgi. There may exist other biosynthetic pathways of SM not reflected by the NBD studies.

The present studies offer insight into a pool of SM involved in signaling. This pool of SM is characterized by resistance to bSMase, delayed labeling kinetics, and solubilization by Triton X-100. Since the hormone-activated neutral sphingomyelinase localizes to the cytosol and the product of SM hydrolysis, choline phosphate, is released intracellularly and not into the medium, these results taken together point strongly to an intracellular localization of the signaling pool of SM. The Percoll fractionation studies demonstrate that this pool co-fractionates with the plasma membrane fraction and possibly the Golgi. Therefore, this signaling pool may reside in the inner leaflet of the plasma membrane or on the cytoplasmic face of a subcellular fraction (such as Golgi or endosomes) that co-fractionates with the plasma membrane. Also, many of the lipid-regulated enzymes and lipases participating in signal transduction pathways appear to localize to the cytosol and/or associate with the inner leaflet of the plasma membrane. These enzymes, which include protein kinase C, phospholipase C, and phospholipase A₂, interact with lipid activators or substrates, which are suggested to localize to the inner leaflet of the plasma membrane. In addition, we have recently identified a ceramide-activated protein phosphatase that fractionates in the cytosolic fractions in leukemia and glioma cells. Therefore, the target of the generated ceramide also suggests an intracellular site of operation of the SM cycle.

The intracellular location of the SM signaling pool bears two important implications. The first implication is regarding the function of intracellular SM. While it was previously assumed that intracellular SM represented a transit population of newly synthesized lipid en route for eventual delivery to the plasma membrane outer leaflet, there may be more than a single SM population (such that one subpopulation of SM may indeed be dedicated toward maintaining the plasma membrane pool, while another subpopulation may be dedicated for functioning in signal transduction). A second implication concerns the metabolism of the signaling SM pool. The two pools of SM identified by bSMase exhibit distinct differences in their labeling kinetics such that the signaling pool (which is bSMase-resistant) shows delayed kinetics of labeling. This difference in labeling raises the important question of the identity of the enzymes and metabolic pathways involved in the biosynthesis of the signaling pool of SM. The outer leaflet pool is thought to be created by the fusion of vesicles derived and delivered from the Golgi apparatus (which contain the newly synthesized SM on the luminal face) with the plasma membrane, rendering the SM facing on the outer leaflet. Is the inner leaflet pool derived from the outer leaflet pool, or is it synthesized in a metabolically independent manner? If the intracellular SM is derived from the outer leaflet then the SM must be flipped into the inner leaflet. However, there is no evidence of SM flipase at the plasma membrane, and spontaneous flipping times are on the order of days (27). On the other hand, there have been reports of de novo SM synthesis at the plasma membrane (53, 54). This or a related synthetic activity may generate and replace the SM of the inner leaflet. Along the same theme, this synthetic activity, which is distinct from the synthetic activity of the Golgi lumen, may play a specific role in signal transduction.

In conclusion, we have identified at least two distinct pools of SM in human leukemia cells. We suggest that the pool involved in the SM cycle of signal transduction is localized to the intracellular portion of the cell, most likely to the inner leaflet of the plasma membrane. In a broader scope, this broaches the idea that cells contain distinct and unique pools of lipids that may be biochemically, metabolically, and functionally distinct.

Acknowledgments—We thank Supriya Jayadev for careful review of this manuscript, and Marsha Haigood for expertsecretarial assistance.

REFERENCES

1. Callis, P. R., and Hope, M. J. (1980) Biochim. Biophys. Acts 597, 533–542.
2. Van Blitterswijk, W. J., Van der Meer, B. W., and Hilkmann, H. (1987) Biochemistry 26, 1745–1756.
3. Brown, D. A., and Rose, J. K. (1992) Cell 68, 533–544.
4. Hannun, Y. A., and Bell, R. M. (1989) Adv. Lipid Res. 25, 27–41.
5. Hannun, Y. A. (1994) J. Biol. Chem. 269, 3123–3128.
6. Okazaki, T., Bell, R. M., and Hannun, Y. A. (1989) J. Biol. Chem. 264, 19076–19080.
7. Kim, M.-Y., Linnardc, C., Obeid, L., and Hannun, Y. Y. (1991) J. Biol. Chem. 266, 484–489.
8. Dressler, K. A., Mathian, S., and Kolesnich, R. N. (1992) Science 255, 1715–1718.
9. Mathias, S., Younes, A., Kan, C.-C., Orlow, I., Joseph, C., and Kolesnich, R. N. (1993) Science 259, 519–522.
10. Alldice, L. R., Chao, C. P., Hölzner, M. A., Barker, S. C., and Raghov, R. (1992) J. Biol. Chem. 267, 20044–20050.
11. Alldice, L. R., Barker, S. C., Polestheiwane, A. E., and Kang, A. H. (1990) J.
