Sphingomyelinase D, a novel probe for cellular sphingomyelin: effects on cholesterol homeostasis in human skin fibroblasts

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Abstract **Sphingomyelin (SM) and free cholesterol (FC) are concentrated in the plasma membranes of eukaryotes; however, the physiological significance of their association is unclear. A common tool for studying the role of membrane SM is digestion with bacterial sphingomyelinase (SMase) C, which hydrolyzes SM to ceramide. However, it is not known whether the observed effects of SMase C treatment are due to the loss of SM per se or to the signaling effects of ceramide. In this study, we tested SMase D from *Corynebacterium pseudotuberculosis*, which hydrolyzes SM to ceramide phosphate, as an alternative probe. This enzyme specifically hydrolyzed SM in fibroblasts without causing accumulation of ceramide. Treatment of fibroblasts with SMase D stimulated translocation of PM FC to intracellular sites by <20% of the rate observed after SMase C digestion. The cells regenerated SM nearly completely within 5 h after SMase C treatment. However, even after 20 h, no regeneration occurred following SMase D digestion. These findings suggest that the translocation of PM FC caused by SMase C digestion is due to the cellular effects of ceramide rather than the loss of SM. Since ceramide phosphate does not appear to have such effects, we suggest that SMase D is a useful probe of membrane SM.**—Subbaiah, P. V., S. J. Billington, B. H. Jost, J. G. Songer, and Y. Lange. **Sphingomyelinase D, a novel probe for cellular sphingomyelin: effects on cholesterol homeostasis in human skin fibroblasts.** ***J. Lipid Res.*** **2003.** 44: **1574–1580.**

Supplementary key words ceramide • ceramide phosphate • acyl-CoA:cholesterol acyltransferase • cholesterol efflux • sphingomyelin • sphingomyelinase

Several studies have shown that the bulk of cellular free cholesterol (FC) and sphingomyelin (SM) are localized in the plasma membrane (1–3). These two lipids interact strongly with each other in the membrane through hydrogen bonding and van der Waal's interactions (4, 5). It is therefore not surprising that each influences the intracellular transport and metabolism of the other. For example, depletion of membrane SM by enzymatic degradation leads to a rapid translocation of FC from the plasma membrane to intracellular sites, where it is esterified to cholesteryl ester (CE) by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) (2, 5, 6). Degradation of membrane SM also dramatically increases the oxidizability of membrane cholesterol by bacterial cholesterol oxidase (7), and blocks the proteolysis of sterol regulatory element binding protein, thereby inhibiting cholesterol biosynthesis (8). Conversely, exogenous addition of SM to human skin fibroblasts stimulates the biosynthesis of cholesterol (9). Ohvo, Olsio, and Slotte (10) reported that SM depletion of fibroblasts results in increased efflux of membrane FC to 2-hydroxypropyl-β-cyclodextrin (HPCD). In all these studies, the depletion of membrane SM was achieved by enzymatic degradation using bacterial sphingomyelinase (SMase) C, which hydrolyzes SM to ceramide and phosphorylcholine. Since ceramide is a strong mediator of signal transduction reactions (11–13), it is not clear whether the various effects observed following SMase C treatment are due to the disruption of the SM-cholesterol interaction and the consequent loss of cholesterol from the membrane, or rather to the effects of ceramide on cellular metabolism. A study in Chinese hamster ovary (CHO) cells showed that short-chain ceramides are strong inhibitors of ACAT activity (14). However, natural long-chain ceramides had no effect on the enzyme activity. C2 ceramides have also been reported to inhibit the synthesis of phosphatidylcholine (PC), sphingolipids, and cholesterol in baby hamster kidney cells, whereas the elevation of natural ceramides in the cells had very little effect on lipid synthesis (15). It has been suggested that the generation of ceramide disrupts the membrane bilayer because of the formation of hexagonal II phases (12). Such local phase transitions may cause profound changes in

Manuscript received 6 March 2003, and in revised form 15 May 2003.

Published, JLR Papers in Press, June 1, 2003.
DOI 10.1194/jlr.M300103-JLR200

Abbreviations: CE, cholesteryl ester; FC, free cholesterol; HPCD, 2-hydroxypropyl-β-cyclodextrin; PC, phosphatidylcholine; PKC, protein kinase C; SM, sphingomyelin; SMase, sphingomyelinase.

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the activities of membrane enzymes and receptors, in addition to independently affecting the retention of membrane cholesterol. Here we investigated the effect of depletion of SM without the generation of ceramide, utilizing a SMase from *Corynebacterium pseudotuberculosis* (SMase D) that specifically hydrolyzes SM to ceramide phosphate and free choline (16). Since the ceramide phosphate generated in this reaction is a phospholipid, it probably is less disruptive to membrane structure than is ceramide. Furthermore, unlike ceramide, ceramide phosphate is not known to have significant signal transduction effects, although the short-chain ceramide phosphate analogs have been reported to stimulate DNA synthesis and to antagonize the effects of short-chain ceramides (17). The results presented here show that, for equal degradation of membrane SM, treatment with SMase D indeed does have a significantly smaller effect on cholesterol mobilization than that observed with SMase C. In light of these findings, many of the previous studies that employed SMase C to study the role of membrane SM in cellular functions may need to be reexamined. We suggest that SMase D is a novel and less disruptive probe than SMase C for the study of membrane SM.

EXPERIMENTAL PROCEDURES

Materials

SMase C from *Staphylococcus aureus* (121 U/mg), HPCD, and C-2 and C-6 ceramides were purchased from Sigma. [Oleoyl-1-^{14}C]CoA was purchased from Dupont NEN and [methyl-^{14}C]choline was obtained from American Radiolabeled Chemicals, Inc. [1α, 2α-^{3}H]cholesterol was purchased from Amersham Pharmacia Biotech. Histidine-tagged *C. pseudotuberculosis* SMase D was expressed in *Escherichia coli* from plasmid pJGS91, which contains the coding region for a six-histidine tag supplied by the vector, pTrcHisB. The enzyme was purified by affinity chromatography on Talon resin (Clontech), as described previously (18). The specific activity of the enzyme was 24.5 nmol of SM hydrolyzed/h/μg protein using egg SM liposomes as substrate. The activity of the enzyme on cell membranes was significantly higher than on liposome substrates (results not shown). The substrate specificity of SMase D was confirmed from the hydrolysis of erythrocyte and fibroblast phospholipids. Only SM was hydrolyzed in both cell membranes, and approximately stoichiometric amounts of ceramide phosphate were formed, showing that very little phosphatase activity was present. Ceramide phosphate was prepared from the hydrolysis of egg SM using purified SMase D, and purified by preparative TLC on silica gel plates using the solvent system chloroform-methanol-water (65:25:4, v/v/v).

Cell culture

Human foreskin fibroblasts (1) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. The viability of cells following SMase treatment was measured from the determination of lactate dehydrogenase (LDH) in the medium (19).

Assay of in vivo cholesterol esterification

Cells were incubated for 10 min at room temperature in PBS containing [^{3}H]cholesterol (1 μCi) solubilized in 20% HPCD (final concentration 0.05%). The labeled buffer was removed, the cell monolayer rinsed, and DMEM containing 5% lipoprotein-deficient serum added to the flasks. Enzymes or ceramides were added and the cells incubated for the times indicated in the figure legends. Cell extraction and assay of the esterification of [^{3}H]cholesterol were as described (20). The fractional rate of plasma membrane cholesterol esterification was determined from the radioactivity in free and esterified cholesterol as: [^{3}H]CE/[^{3}H]CE + [^{3}H]cholesterol).

ACAT activity

The activity of ACAT in vitro was assayed as described (21). Briefly, cells were dissociated, washed, and allowed to swell for 10 min on ice in 0.25 M sucrose + 5 mM sodium phosphate (pH 7.5) prior to homogenization by eight to ten strokes in a mechanical homogenizer. After a low-speed spin to remove nuclei and intact cells, ceramide, excess cholesterol solubilized in Triton WR-1339, and 350 μM [^{14}C]oleoyl CoA were added to aliquots of the supernatant. The mixtures were incubated for 20–30 min at 37°C. Extraction of lipids and determination of radioactivity were as described (21).

Phospholipid and protein estimation

Phospholipids were separated on silica gel TLC plates using the solvent system chloroform-methanol-acetic acid-0.15 M NaCl (60:30:10:3, v/v/v/v). The spots (in ascending order) corresponding to lyso PC, SM, PC, phosphatidylinositol (PI) + phosphatidylserine (PS), phosphatidylethanolamine (PE), and ceramide phosphate (when present) were scraped, and their phosphorus content estimated using the modified Bartlett procedure (22). Protein was estimated by the BioRad procedure (23) using BSA as standard.

Kinetics of efflux of [^{3}H]cholesterol

Cells in 75 cm^2 flasks were incubated in PBS with ~0.6 μCi [^{3}H]cholesterol in 0.05% HPCD for 15 min at room temperature. The medium was then removed, the cells rinsed with PBS, dissociated with trypsin, resuspended in growth medium, plated in 25 cm^2 flasks, and allowed to reattach. (This replating minimized contamination by the extracellular [^{3}H]cholesterol adsorbed to the plastic in the first flask.) For the measurement of cholesterol efflux, the cells were incubated at 37°C with 0.13–0.20% HPCD in PBS. The buffer was replaced at frequent intervals (3–8 min) to make the efflux of label irreversible. Aliquots of the buffer at each time point were centrifuged and counted. At the end of the time course, the cells were assayed for [^{3}H]cholesterol, phospholipid, and protein. The data were expressed as the percentage of total radioactivity transferred to the medium.

Regeneration of SM

Fibroblasts were grown in 35 mm wells and labeled with [^{14}C]choline (1 μCi/ml) in serum-free DMEM for 60 h. The cells were then washed with PBS, and serum-free medium containing SMase C (0.12 U/ml) or SMase D (0.1 μg/ml) was added. After incubation for 1 h, the medium was replaced with fresh serum-free medium, and the cells were incubated for 18 h at 37°C. Duplicate wells were processed at the indicated times, and the radioactivity in cellular PC and SM determined following their separation on TLC plates using the solvent system chloroform-methanol-water (65:25:4, v/v/v).

RESULTS

Specificity of SMase C and SMase D

We first investigated the specificity of the two SMases with respect to the hydrolysis of cellular phospholipids in intact cells. Fibroblasts were grown to confluency and incubated with either SMase C or SMase D for 2 h at 37°C.

Total cell lipids were extracted, and the phospholipids separated on a silica gel TLC plate and quantitated from lipid phosphorus. Under these conditions, both enzymes hydrolyzed SM only (**Fig. 1**). Although previous studies showed that lyso PC is also hydrolyzed by SMase D (24), this was not the case in our system, presumably because cellular lyso PC is not exposed to the enzyme in intact cells. About 60% of the membrane SM was hydrolyzed by each enzyme in our experimental conditions. The cells appeared normal under the light microscope, and no cells had detached after 2 h of treatment.

The effect of vigorous SMase treatment on cell integrity was determined from the release of the cytosolic enzyme, LDH, into the medium. After hydrolysis of 63% and 64% of cell SM by SMase C and SMase D, respectively, 6% and 7% of cell LDH was released in 2 h compared with 7% in untreated cells.

There was accumulation of near-stoichiometric amounts of ceramide phosphate in the cells treated with SMase D (**Table 1**), suggesting that ceramide phosphate was not hydrolyzed further in fibroblasts. These results demonstrate that SMase D treatment depletes membrane SM in living cells without damaging the cells, and without the accumulation of ceramide.

Effect of SMases on FC mobilization

Translocation of cell surface cholesterol to the endoplasmic reticulum was assayed from its esterification. Fibroblasts were pulse-labeled with [^{3}H]FC, and were treated with either SMase C or SMase D for 1.5 h to 2 h at 37°C.

TABLE 1. Accumulation of ceramide phosphate in cells following sphingomyelinase D treatment

Experiment	Decrease in SM	Increase in Cer P	Recovery
	nmol/mg protein		*%*
1	32.3	31.3	96.9
2	28.0	26.6	94.8
3	31.5	29.9	94.9
4	15.6	18.2	116.6

Cer P, ceramide phosphate; SM, sphingomyelin. Fibroblasts in 75 cm^2 flasks were treated with sphingomyelinase D for 60 min, and the phospholipids were separated on TLC plates and the lipid phosphorus determined, as described in the text.

The lipids were extracted and analyzed to determine the percent of labeled cholesterol esterified (20). In agreement with previous studies (5), treatment with SMase C increased the esterification of membrane FC about 4.7-fold compared with untreated cells (**Fig. 2A**). However, treatment with SMase D increased esterification only 1.7-fold. Degradation of cell SM was comparable for both SMases, as measured by the SM/PC ratios (Fig. 2B). Thus, for equal SM degradation, SMase C causes significantly more esterification of plasma membrane FC than does SMase D.

Effect of ceramides and ceramide phosphate on esterification of plasma membrane cholesterol

A possible explanation for the stimulation of plasma membrane cholesterol esterification following SMase C treatment is that the ceramide generated by the enzyme stimulates either the translocation or the esterification of

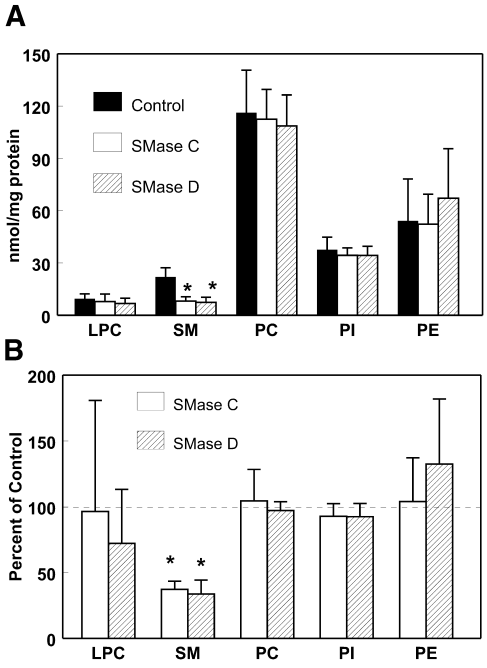

Fig. 1. Specificity of sphingomyelinases (SMases) C and D. Fibroblasts were treated with SMase C (0.12 U/m) or SMase D (0.1 μg/ml), for 2 h at 37°C, as described in Experimental Procedures. Total lipids were extracted, and the phospholipids were analyzed by TLC and lipid phosphorus assay. The values shown are mean ± SD of four separate experiments. A: The concentration of individual phospholipids. B: The values after SMase treatment, expressed as percent of control.

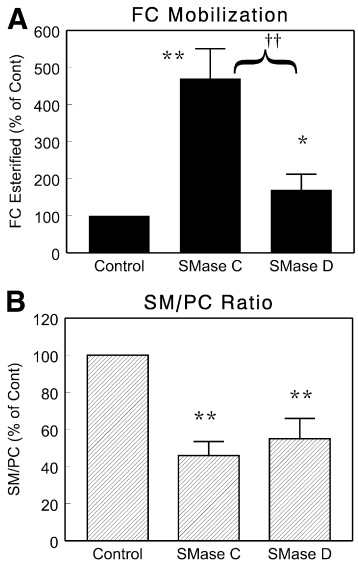

Fig. 2. Effect of sphingomyelin (SM) depletion by SMase C and SMase D on cholesterol mobilization from the plasma membrane. Fibroblasts were labeled with [^{3}H]free cholesterol (FC), and then treated with either SMase C (0.12 U/ml) or SMase D (0.1 μg/ml) for 2 h at 37°C. The transfer of labeled FC from the plasma membrane to the ER was assessed from its esterification, as described in Experimental Procedures. A: The percent of labeled FC esterified [100× cholesteryl ester (CE)/(FC+CE)]. B: The SM/phosphatidylcholine (PC) ratio at the end of the incubation. *P* values: * <0.05 versus Control; ** <0.02 versus Control; †† <0.01 SMase C versus SMase D.

FC by ACAT. To examine these alternatives, we tested the effect of two short-chain ceramides (C2 and C6) that have been widely used as surrogates for the natural ceramides because they are cell permeable. Fibroblasts labeled with [^{3}H]FC were incubated with varying concentrations of ceramides, and the esterification of FC was determined after 2 h. Since the cells were not treated with SMase, the membrane SM was not altered, and the basal rate of cholesterol esterification was low in these experiments. As shown in **Fig. 3**, esterification was inhibited by both ceramides even at concentrations lower than those normally used for inducing cell differentiation (25), apoptosis (15), or cell growth (26). Similar inhibition of esterification by short-chain ceramides has been reported in CHO cells (14). Addition of long-chain ceramide (derived from brain SM) did not have any effect on esterification (results not shown). These findings suggest that the increased esterification of membrane FC in the presence of SMase C is not the result of the direct stimulatory effect of ceramide on ACAT activity.

We next studied the effect of the product of SMase D, ceramide phosphate, on FC esterification in intact cells. This compound also inhibited esterification (**Fig. 4**), although the effect was much smaller than that observed with short-chain ceramides. Since the ceramide phosphate used here is the long-chain compound derived from natural SM, it is unlikely that it traversed the plasma membrane.

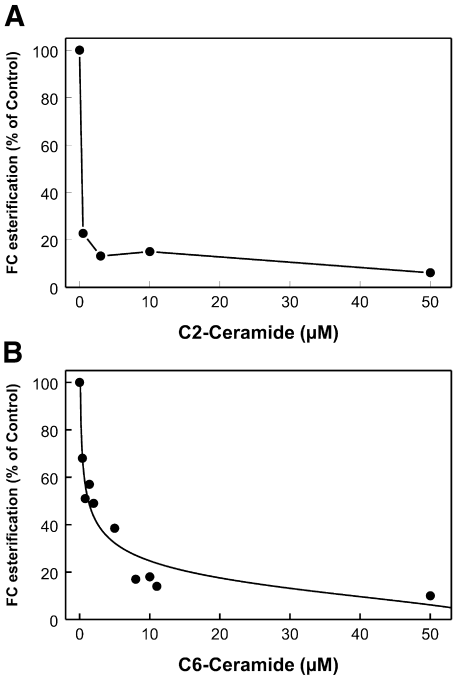

Fig. 3. Effect of exogenous short-chain ceramides on the esterification of plasma membrane cholesterol in intact cells. Fibroblasts were labeled with [^{3}H]FC and then incubated for 2 h at 37°C with the indicated concentrations of C2-ceramide (A) or C6-ceramide (B). Esterification of labeled FC was determined as described in Experimental Procedures, and expressed as a percentage of that in untreated cells.

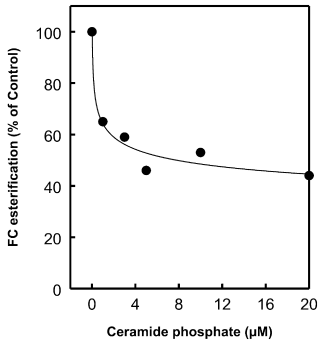

Fig. 4. Effect of ceramide phosphate on esterification of plasma membrane cholesterol in intact cells. The experiment was performed as described in the legend to Fig. 3, except that long-chain ceramide phosphate (derived from egg SM) was added to the medium.

Effect of SMase products on ACAT activity in a cell-free system

We examined the effects of ceramides and ceramide phosphate on ACAT activity in cell homogenates in the presence of excess cholesterol. Both C2 and C6 ceramides inhibited ACAT activity (**Fig. 5**), although the extent of inhibition was lower than that observed in intact cells. Ceramide phosphate also inhibited ACAT in cell-free homogenates, but much less than the ceramides (results not shown).

Effect of SMases on cholesterol efflux

Previous studies showed that SMase C treatment not only increases the movement of plasma membrane FC to intracellular compartments, but also increases efflux to an extracellular acceptor (10). We therefore compared the efflux of labeled FC to cyclodextrin in cells treated with SMase C and SMase D. The cells were pulse labeled with [^{3}H]cholesterol, and the transfer of label to HPCD determined at 37°C. In control cells, ~16% of the cellular [^{3}H]cholesterol was released from the cells in 30 min (**Fig. 6**). However, neither SMase C nor SMase D in-

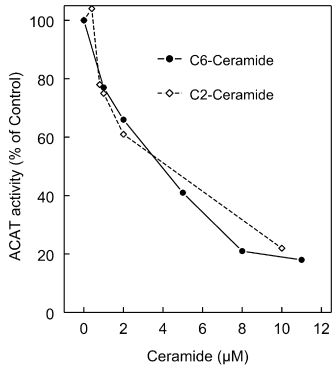

Fig. 5. Effect of C-6 ceramide and long-chain ceramide phosphate on ACAT activity in cell-free homogenates. Fibroblast homogenates were prepared as described in Experimental Procedures. Aliquots were incubated for 20 min at 37°C with [1-^{14}C]oleoyl CoA and C-6 ceramide, ceramide phosphate, or solvent alone. The fraction of cholesterol esterified in vitro was calculated from the amount of ^{14}C incorporated into esters and the specific activity of [^{14}C]oleoyl CoA. Values are expressed relative to a solvent control.

creased the efflux appreciably. Similar results were obtained when the cells were labeled with [^{3}H]cholesterol for 48 h in growth medium prior to the enzyme treatment. Furthermore, there was no stimulation of efflux by either SMase C or SMase D when apolipoprotein A-I (apoA-I)/egg PC complex was used as acceptor (results not shown). Our findings do not agree with those of Ohvo, Olsio, and Slotte, who reported that SMase C treatment of baby hamster kidney fibroblasts led to stimulation of the transfer of plasma membrane cholesterol to exogenous cyclodextrin (10); however, they are in accord with previous studies that showed that SMase C treatment of human skin fibroblasts, macrophages, and smooth muscle cells stimulated cholesterol esterification but not efflux (27).

Regeneration of SM

It has been shown that the SM hydrolyzed by SMase C is regenerated at the expense of cellular PC within a few hours of treatment (10, 28). To determine whether similar regeneration occurs after SMase D treatment, we labeled fibroblasts with [methyl-^{14}C]choline for 60 h, and treated them with either SMase C or SMase D for 1 h. There was an ~50% reduction in cellular SM label with both treatments. The cells were then incubated in serum-free medium for up to 20 h, and the label in PC and SM was determined. In cells treated with SMase C, SM levels returned to near control levels within 5 h, while the label in PC decreased (**Fig. 7**). However, in cells treated with SMase D, there was very little regeneration of labeled SM even after 20 h. Thus, the cells apparently do not restore SM after its degradation to ceramide phosphate. These results confirm that ceramide phosphate is not hydrolyzed to ceramide under the conditions of the experiment. Inclusion of 10 μM C2 ceramide in the medium of SMase D-treated cells did not lead to formation of labeled SM, suggesting that the ceramide has to be generated in situ for the regeneration of SM to occur (results not shown). The decrease in PC radioactivity was comparable in the two treatment groups, indicating that it is due to the normal turnover of membrane PC. The total label in PC was 10 times greater than that in SM. Thus, only a fraction of the PC label lost accounts for the replenishment of SM in the SMase C-treated cells. The levels of another choline-containing phospholipid, lyso PC, were not significantly affected by treatment with either enzyme (results not shown).

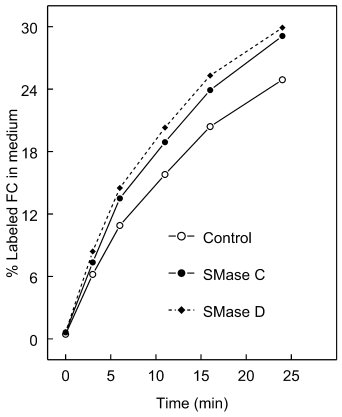

Fig. 6. Effect of SMase C and SMase D on cholesterol efflux. Cells were labeled with [^{3}H]cholesterol, and treated with SMase C (closed circle), SMase D (triangle), or solvent alone (<1% ethanol) (open circle) and incubated with 0.15% 2-hydroxypropyl-β-cyclodextrin in PBS as described in Experimental Procedures. Aliquots of the medium were removed at the times indicated and counted for ^{3}H. The ^{3}H left in the cells at the end of the incubation was also measured. Transfer is expressed as 100 × [^{3}H]dpm in medium/([^{3}H]dpm in cell + [^{3}H]dpm in medium) for each time point.

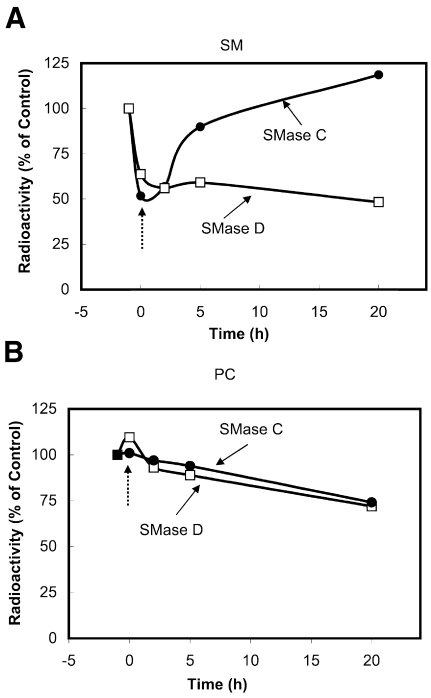

Fig. 7. Regeneration of SM after SMase treatment. Replicate wells of fibroblasts were labeled with [^{14}C]choline for 60 h in serum-free medium and then treated for 1 h with either SMase C or SMase D in fresh serum-free medium. The cells were washed with PBS and incubated in serum-free medium for an additional 20 h. At the times indicated, cells were assayed for the radioactivity in choline-containing lipids as described in Experimental Procedures. The end of the enzyme treatment period is indicated by the dotted arrow. A: The radioactivity in SM. B: The radioactivity in PC.

DISCUSSION

SM is one of the most abundant phospholipids in the plasma membrane of eukaryotes. Its specific localization in the outer monolayer and its role in raft formation are well established (29, 30). However, the functional significance of its localization and its specific association with FC in the membrane are incompletely understood, partly because of the limited tools available for modifying membrane SM. The most widely used method to deplete membrane SM uses bacterial SMase C, which hydrolyzes SM to ceramide and choline phosphate. The ceramide generated by this enzyme has profound effects on signaling pathways (12, 13). Furthermore, the ceramide is rapidly

converted back to SM at the expense of membrane PC. Diacylglycerol, a product of the regeneration reaction, is a potent activator of protein kinase C (PKC) (31). Moreover, commercial preparations of SMase C may be contaminated with phospholipase C activity because the two enzymes coexist in the bacterial source. As a result, it is difficult to differentiate between the effects of a decrease in membrane SM and the actions of ceramides and diacylglycerols formed during the reaction. Since a recent study implicated PKC in the translocation of membrane cholesterol (32), it is important to distinguish the effects of diacylglycerol from those of SMase. We have employed a SMase that hydrolyzes SM to ceramide phosphate and free choline. To be consistent with phospholipase terminology, this enzyme should therefore be called SMase D. This enzyme is the toxic agent produced by *C. pseudotuberculosis* that causes caseous lymphadenitis in sheep and goats (33). A similar enzyme is found in the venom of the brown recluse spider (18), but it has not been identified in mammalian systems. It specifically hydrolyzes cell surface SM in fibroblasts, apparently without affecting cell viability. Experiments with CHO cells and human astrocytes gave similar results (not shown). Ceramide phosphate was not hydrolyzed to ceramide by the cells, as evidenced from its stoichiometric accumulation. Our use of the recombinant enzyme ensured that there was no contamination with other phospholipases. We propose that this enzyme provides a useful alternative to SMase C for the study of the role of plasma membrane SM in cellular functions.

Although both enzymes hydrolyzed comparable quantities of SM, the stimulation of plasma membrane cholesterol movement to the ER after SMase D treatment is only about 20% of that seen after SMase C treatment. This suggests that the increase in cholesterol movement induced by SMase C is due either to the effects of the ceramide formed or to the inability of ceramide-rich membranes to retain cholesterol. While we could not determine the effect of long-chain ceramides, the first possibility is not directly supported by the finding that exogenous ceramide analogs inhibit ACAT activity (Figs. 3, 5). The observed difference between the two SMases could be due to the effects of their different products on the structure of the membrane bilayer. In contrast to ceramide, which is known to induce hexagonal phases (12), ceramide phosphate may favor the formation of lamellar structures similar to the parent SM molecule. The interaction of cholesterol with SM is due to hydrogen bonding and van der Waal's forces that are dependent on the NH, CO, and the OH functions of SM (5, 34–36), all of which are retained in both ceramide and ceramide phosphate. However, since ceramide does not form lamellar structures, it is possible that the interaction with cholesterol is weak, and consequently the sterol is more likely to leave the plasma membrane. On the other hand, ceramide phosphate, by virtue of maintaining the bilayer structure, retains cholesterol in the membrane. Zha et al. (37) reported that SMase C treatment of macrophages and fibroblasts induces the formation of vesicular structures from the plasma membrane. It is possible that cholesterol is transported in such vesicles to the ER and other intracellular sites. Vesicular structures may not form after SMase D treatment, possibly accounting for the observed differences in FC mobilization. Recent studies with SM-deficient cell lines show that the presence of SM is not essential for the distribution of cholesterol to the plasma membrane (38). This also suggests that the translocation of cholesterol from the plasma membrane to the ER in response to SMase C treatment is not due to the depletion of SM per se, but due to the in situ generation of ceramide, and consequent disturbance of the bilayer. Exogenous short-chain ceramides cannot reproduce this effect, perhaps because they are not retained in the membrane, but enter the cell where they inhibit ACAT activity directly.

The SM hydrolyzed by SMase C action is regenerated rapidly by the SM synthase-catalyzed transfer of phosphorylcholine from PC (10, 28). However, it should be noted that the regeneration of SM is accompanied by the formation of equimolar amounts of diacylglycerol, a potent activator of protein kinases (31, 39). Furthermore, diacylglycerol and other PKC activators stimulate the translocation of intracellular cholesterol to the plasma membrane, whereas PKC inhibitors reduce it (40). Certain PKC activators increase the pool of FC in the endoplasmic reticulum, while some PKC inhibitors decrease it (32). The possible importance in cholesterol homeostasis of diacylglycerol generated by SM regeneration remains to be investigated. A recent study suggests that the increase of diacylglycerol may be essential for the increased intracellular trafficking of cholesterol that accompanies apoA-I-mediated lipid efflux (41).

We found that, in contrast to the cells treated with SMase C, those treated with SMase D do not regenerate SM even after 20 h. This suggests that: *1*) ceramide phosphate is not converted to ceramide under these conditions; *2*) the cells cannot synthesize SM directly from ceramide phosphate; and *3*) any membrane perturbation due to SMase D digestion may not be severe enough to trigger repair mechanisms in the cell. Since SM regeneration following SMase C digestion occurs at the expense of PC, leading to diacylglycerol production, the possible effect of diacylglycerol in downstream events such as activation of protein kinases can be avoided by the use of SMase D. The relatively mild effects of SMase D are also evident from a recent study in human neutrophils that showed that, while SMase C treatment of the cells induced an increase in intracellular calcium and dramatic degranulation, treatment with brown recluse spider venom, which contains SMase D, had no such effects, although the hydrolysis of SM was comparable (42). In summary, we have described a new probe for studying the role of cellular SM. The advantages of this probe over SMase C include: *1*) ceramide and diacylglycerol, both potent signaling molecules, are not formed in the reaction with SMase D; *2*) ceramide phosphate, a phospholipid generated by SMase D, is less disruptive to the membrane than the ceramide generated by SMase C; and *3*) there are several endogenous SMases C, which can complicate the interpretation of the effects of exogenous SMase C, whereas there is no known SMase D in mammalian cells. Therefore,

the effects observed can be attributed entirely to the action of the exogenous enzyme.

This study was supported by National Institutes of Health Grants HL-68585, HL-52597 (P.V.S.), and HL-28448 (Y.L.). The authors wish to acknowledge the excellent technical assistance of Jin Ye, Susan Sage, and Jennifer Sowa. The authors thank Robert Sargis for help in the purification of SMase D.

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