Probing compartment-specific sphingolipids with targeted bacterial sphingomyelinases and ceramidases

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Running title: Analysis of compartment-specific sphingolipids

**Abbreviations:** aCDase: acid ceramidase. ACER: alkaline ceramidase. bCDase: bacterial ceramidase. bSMase: bacterial sphingomyelinase. Cer: ceramide. CerS: ceramide synthase. CERT: ceramide transfer protein. DAG: diacylglycerol. DAPI: 4',6-diamidino-2-phenylindole. dhCer: dihydroceramide. ER: endoplasmic reticulum. GlcCer: glucosylceramide. GSL: glucosylsphingolipid. KDS: 3-ketodihydrosphingosine. nCDase: neutral ceramidase. PC: phosphatidylcholine. SL: sphingolipid. SM: sphingomyelin. SMS: sphingomyelin synthase. Sph: sphingosine. SPT: serine palmitoyltransferase. S1P: sphingosine 1-phosphate. TGN: trans-Golgi network. TNF: tumor necrosis factor.
ABSTRACT

Sphingolipids contribute to the regulation of cell and tissue homeostasis, and disorders of sphingolipid metabolism lead to diseases such as inflammation, stroke, diabetes and cancer. Sphingolipid metabolic pathways involve an array of enzymes that reside in specific subcellular organelles, resulting in the formation of many diverse sphingolipids with distinct molecular species based on the diversity of the ceramide structure. In order to probe compartment-specific metabolism of sphingolipids, in this study, we analyzed the ceramide and sphingomyelin species preferentially produced in inner plasma membrane, Golgi apparatus, endoplasmic reticulum, mitochondria, nucleus and cytoplasm by using compartmentally-targeted bacterial sphingomyelinases and ceramidases. The results showed that the length of the acyl chain of ceramide becomes longer according to the progress of ceramide from synthesis in the ER to the Golgi apparatus, then to the plasma membrane. These findings suggest that each organelle shows different properties of sphingomyelin-derived ceramides, consistent with their emerging distinct functions in vitro and in vivo.

Keywords: ceramide molecular species, sphingosine, sphingomyelin, mass spectrometry, cellular organelles.
INTRODUCTION

Sphingolipid metabolism occurs in all eukarya and also in some bacteria such as *Sphigomonas* genus (1). Sphingolipids play important structural roles in cell membranes, as well as, key biologic functions, such as signal transducers and regulatory molecules (2).

The biosynthesis and intraconversion of sphingolipids occur in many subcellular compartments. Starting in the endoplasmic reticulum (ER), the first step in the sphingolipid metabolic pathway is the condensation of L-serine and palmitoyl-CoA catalyzed by serine palmitoyltransferase (SPT) to generate 3-ketodihydrosphingosine (KDS) in the *de novo* pathway (3). KDS is subsequently reduced to form dihydrosphingosine (sphinganine) which is then N-acylated by (dihydro)-ceramide synthases (CerSs) to produce dihydroceramide (dhCer) or ceramide (Cer) still in the ER. CerS activity has also been detected in mitochondrial fractions (4, 5). ER-Cer can then be shipped to the Golgi apparatus by two mechanisms, directly transported by a Cer transport protein and by vesicle transport. In the Golgi, Cer can be phosphorylated by Cer kinase, glycosylated by glucosyl or galactosyl ceramide synthases. Another pathway for the Golgi metabolism of Cer is sphingomyelin synthase (SMS) action which utilizes a phosphocholine headgroup from phosphatidylcholine (PC) in the biosynthesis of sphingomyelin (SM) (5-7). In the *cis*-Golgi compartment, SMS1 and SMS2 use Cer and PC as substrates to produce SM, thereby releasing diacylglycerol (DAG) (8). SMS2 is also localized in the plasma membrane. Alternatively, Cer is converted to glucosylceramide (GlcCer) in the *cis*-Golgi which can be delivered to the *trans*-Golgi network (TGN) for the synthesis of more complex glycosphingolipids (7).

Sphingolipid catabolism is conducted by a series of hydrolases that act on complex GSLs (cerebrosidases) and on SM (sphingomyelinases) resulting in the formation of Cer which is further hydrolyzed to sphingosine (Sph). As such, Sph is only formed from Cer hydrolyzed by ceramidases, and Sph can be phosphorylated by sphingosine kinases to produce sphingosine 1-phosphate (S1P), a key bioactive sphingolipid (9, 10). In turn, action of S1P lyase results in ‘exit’ from the sphingolipid pathway with the formation of fatty aldehydes and ethanolamine phosphate.
Significantly, sphingolipids display compartment-specific metabolism. This is brought home with the identification of the various sphingomyelinases (SMases), cerebrosidases, and ceramidases that reside in distinct compartments. SMases are characterized by pH optima for their enzymatic activity into acidic, neutral, and alkaline SMases and sub classified based on requirement for divalent cations (11). Acid SMase (aSMase) exists in two forms: a zinc (Zn (2+))-independent lysosomal aSMase (L-SMase) and a Zn (2+)-dependent secreted aSMase (S-SMase). Among the four vertebrate neutral SMases (nSMases), nSMase1 is located in ER, and nSMase2, which is activated by diverse stimuli, including the anionic phospholipid phosphatidylserine, strictly localizes at the plasma membrane (PM) and Golgi (12). nSMase3 has been found in ER, Golgi and on PM, and another nSMase is found in mitochondria (MA-nSMase) (13). Next, ceramidases are responsible for hydrolyzing Cer into Sph and free fatty acids. As with SMases, ceramidases are also divided into three types of acid, neutral and alkaline, based on their pH required for optimal activity (11, 14). Acid ceramidase (aCDase) is located within lysosomes, and is involved in the regulation of cell viability (15). Neutral ceramidase (nCDase) is localized to the PM, mitochondria and Golgi, which is involved in digestion, further it is implicated in colon carcinogenesis (16, 17). Alkaline ceramidases (ACER1, ACER2 and ACER3) are primarily located in ER and Golgi complexes and have several cell regulatory functions (18-20). Therefore, it is important to understand the compartmentalized functions of sphingolipid metabolism in order to understand the overall pathway (21).

Moreover, sphingolipids display significant structural diversity, primarily deriving from the complexity of the Cer structure. Thus, Cer is indeed a family of closely related molecules that differ in the length of the N-acyl chain, the nature of the sphingoid backbone, desaturations, and hydroxylations.

These considerations have provided the background for the ‘Many Ceramides’ hypothesis which posits that distinct Cers, formed in distinct compartments, are regulated in distinct manners and mediate distinct functions (22). This is supported by the distinct functions of the different SMs and
Cers. For example, we have shown that the bacterial SMase specifically targeted to mitochondria caused accumulation of Cer in mitochondria and induced cytochrome c release and cell death, but its targeting to the other intracellular compartments was not effective (23, 24). Similarly, induction or addition of SMase to the cell media to access the outerleaflet of the plasma membrane induced cytoskeletal rearrangements but failed to induce cell death (25, 26). Recently, it has been found that forcing translocation of ER-Cers to mitochondria specifically commits cells to death (27). It has also been shown that endogenous Cer generated by nSMase at PM is restricted to that membrane as it is not available for metabolism by glucosylceramide synthase (GCS) at the Golgi (28). In the Golgi, nSMase2 can be a regulator of autophagy and Golgi-Cers participates in cytoprotective autophagy against starvation (29). In terms of specific Cer species, differential regulation of C16 and C24-Cer formation is involved in distinct aspects of B-cell receptor (BcR)-induced apoptosis (30). Moreover, endogenous C18-Cer generation by CerS1 mediates autophagic cell death, and it induces lethal autophagy via selective targeting of mitochondria by LC3B-II-containing autophagolysosomes (31). Also, it has been reported that activation of aSMase and generation of C16-Cer contributed to TNF-α-induced hepatocyte apoptosis (32). Thus, all of these published results strongly suggest that distinct Cer species in different subcellular compartments have distinct cellular functions.

In this study, we report the development of novel tools to modulate SM and Cer levels at different cellular compartments, mimicking activation of specific endogenous SMases and ceramidases. Thus, by using compartmentally-targeted bacterial SMase and ceramidase we analyzed the change in the sphingolipid profile by HPLC-ESI-MS, measuring for the first time differences in sphingolipid composition based on their subcellular location.
MATERIALS AND METHODS

Reagents

Cell culture medium and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA). Anti-V5 tag antibody and Mito Tracker were obtained from Invitrogen, anti-Giantin antibody was from Covance (Berkeley, CA), anti-Flag antibody and anti-Calreticulin antibody was from Sigma-Aldrich (St. Louis, MO).

Cell culture and plasmid DNA transfection

Human epithelial carcinoma (HeLa) cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s supplemented with 10% fetal bovine serum. All cell lines were grown in a 5% CO2 incubator at 37 °C. DNA transfection was performed using XtremeGENE™ 9 DNA Transfection Reagent in accordance with the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO).

Construction of bSMase and bCDase with targeting signal

The DNA sequence for bSMase and bCDase was previously described by our group (33, 34). The bSMase and bCDase genes with targeting signal sequence (Table 1) were directionally subcloned into pCMV and pEF6 vectors tagged with V5 or Flag. Mitochondria, nucleus and ER constructs were adapted from Life Tech pShooter targeting vectors, cat# V821, V822, V823 respectively. The inner plasma membrane is targeted with a Ras Farnesylation signal (KLNPPDESGPSCMSCKCVLS). The Golgi Apparatus is targeted with a signal from human B1,4-galactosyltransferase (MARLREPPLLSGSAAMPQASLQRARRLLVAVCALHLGVTLVYLYLAGRLSRLPQLVGVS TPLQGGSNNSAIGQSGDLRTGGA). The inactive bSMase mutant (D322A/H323A) and the bCDase mutant (Y484A) were generated using Agilent Technologies QuikChange II SDM Kit, cat# 200523. All constructs were verified by restriction mapping and sequencing. For the generation of bSMase and bCDase overexpressing cells, their amplified genes were directionally subcloned into intermediate vector pENTR-D/TOPO (Life Tech cat#K2400-20). Using Gateway
LR Clonase II Enzyme Mix (Life Tech cat#11791-020), the intermediate was recombined with the Destination Cloning vector pLenti6.3/TO/V5-DEST (Life Tech cat# K531520).

**CDase activity assay**

Cultured cells transfected with the various plasmids were collected and washed with PBS. The protein amounts of collected samples were measured using a Pierce™ BCA Protein Assay Kit from Thermo Scientific (#23225) and used with equal amounts of protein. In 96-well plates, a mixture RBM 14-16 substrate at 20 μM with cell lysates in 0.3% Triton X-100, 75mM NaCl, 12.5 mM phosphate buffer, pH 7.4, was incubated for 3 h in the dark. After that, the reaction was stopped by adding methanol and sodium periodate in 100 mM glycine/NaOH, and incubated for 1 h. The released fluorescence was quantified by a microplate fluorescence reader (λex 360 nm, λem 460 nm). Compound RBM 14-16 was a gift from Prof. Antonio Delgado and Prof. Gemma Fabrias at the RUBAM, CID-CSIC in Barcelona (35).

**SMase activity assay**

Bacterial SMase activity was assayed as described previously using [14C-methyl]-SM as a substrate (36). Briefly, cold SM from bovine brain (Avanti polar lipids) and [14C-methyl]-SM were dried under nitrogen gas and resuspended in Triton X-100 mixed micelles by sonication to generate assay buffer. The final reaction conditions contained 0.1% Triton X-100, 50 mM Tris buffer, pH 7.5, 25 mM MgCl₂ with 5 mol% SM. Cell lysates were added to 100 μL of assay buffer and were incubated for 30 min. Reactions were quenched and extracted by the Bligh and Dyer method. The aqueous phase was transferred to scintillation vials containing scintillation fluid and counted.

**Western blot analysis**

Protein extracts from cells were obtained after harvesting or homogenization with RIPA buffer (with PMSF, orthovanadate and protease inhibitor cocktail from Santa Cruz Biotechnology (Santa Cruz, CA)). Protein concentration was measured using a Pierce™ BCA Protein Assay Kit. Proteins were separated by SDS-PAGE using the Criterion system (BioRad) and immunoblotted.

**Immunofluorescence and confocal microscopy**
Cells were seeded in poly-D-lysine-coated confocal dishes (MatTek Corp., Ashland, MA) and treated and fixed in 4.0% paraformaldehyde solution for 20 min and permeabilized with 0.1% Triton X-100 for 5 min. After blocking with 2% of human serum for 20 min, cells were incubated with primary and secondary antibodies overnight at 4 °C. Cells were observed using a LSM510 META confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany) and a Leica SP8 confocal microscopy system (Leica Microsystems, Wetzlar, Germany)(37).

Measurement of lipids

Lipid analysis was performed as previously described (38). Briefly, cells were collected and sonicated, and sphingolipid species were identified on a Thermo TSQ Quantiva triple quadruple mass spectrometer at the Lipidomics Core at Stony Brook University. Sphingolipids from cellular extracts were normalized to total lipid phosphates present in cells after a Blight and Dyer extraction (39, 40).

Statistical Analysis

Results are expressed as the mean ± SD. For statistical analysis, one-way anova with Tukey post-hoc analysis was used. Dendrogram analysis was performed using MATLAB 2018a.
RESULTS

Activity of targeted bacterial SMase and CDase constructs

In order to dissect compartment-specific sphingolipid metabolism, we constructed compartmentally-targeted (inner PM, Golgi apparatus, ER, mitochondria and cytoplasm) bacterial SMases and CDases based on bSMase from Bacillus cereus and bCDase from Pseudomonas aeruginosa (Table 1). In order to evaluate the functionality of the different compartmentalized constructs, SMase and CDase activity were measured in lysates from HeLa cells after 20 h of transfection with the different constructs. As plotted in figure 1A, all bSMase-targeted constructs resulted in an increase of SMase activity as compared with control cells which have a vector containing V5 or Flag coded sequence (Fig. 1A). SMase activity was highest for the Golgi, ER, and cytoplasmic constructs and lower for inner plasma membrane (iPM), mitochondria, and nucleus. CDase activities in cell lysates were measured by using a fluorescent substrate previously described. The increase of CDase activity was shown in cell lysates from cells transfected with all CDase constructs to similar levels (Fig. 1B). Next, we also confirmed the expression of each compartmentally-targeted SMase and CDase in transfected HeLa cells (Fig. 1C). The magnitude of SMase activities in samples varied depending on each construct due to the expression level of SMase. Also, the intensity of CDase activities was dependent on cellular CDase expression. As a result, all designed constructs were active in transfected cells.

Subcellular localization of targeted SMases and CDases

Next, it became important to determine if SMase and CDase proteins tagged with each targeting signal did localize to the expected compartment. After transfection of SMase and CDase constructs in HeLa cells for 20 h, localization of SMase and CDase was visualized using anti Flag or anti V5 antibody (shown in green color) with organelle markers (Giantin as Golgi marker, Calreticulin as ER marker and Mito Traker as mitochondria marker, shown in red color). The results showed that each of the enzymes was observed in its intended compartment (PM, Golgi, ER, mitochondria,
nucleus and cytoplasm) for both bSMases (Fig. 2A) and bCDases (Fig. 2B). Therefore, these results confirm that the tagged proteins show their activities in the proper organelles.

**Effects of targeted CDases and SMases on sphingolipid levels in each organelle**

In order to figure out the effects of CDase and SMase activities on Sph, S1P, Cer and SM levels in different organelles, the levels of intracellular sphingolipids were determined by HPLC-ESI-MS. Expression of iPM-targeted SMase resulted in an increase of cellular Cer levels, although no significant changes in SM were detected. (Fig. 3A). Expression of iPM-targeted CDase *per se* had no measureable effects on Sph, S1P, Cer or SM. On the other hand, co-expression of iPM-targeted CDase and iPM-targeted SMase decreased the levels of Cer seen with iPM-targeted bSMase, increased the production of Sph further, but had no statistically significant effects on S1P and SM (Fig. 3A). These results confirmed that these constructs were active in the cell, suggesting availability of SM in the inner leaflet of the plasma membrane, and supporting our previous results pointing to no or little Cer present in basal conditions, unless there is an activation of plasma membrane SMase (26).

Next, the change of sphingolipid levels in the Golgi was analyzed (Fig. 3B). Cells transfected with Golgi-targeted SMase showed a significant increase of Cer in parallel with a decrease of SM. Golgi-targeted bCDase had little effects on Sph, Cer, S1P or SM. However, the action of Golgi bCDase in the presence of Golgi-targeted bSMase resulted in attenuation of the Cer levels and in a remarkable formation of Sph (Fig. 3B). These results support the presence of SM in the Golgi and similar to iPM, the absence of Cer in basal conditions.

ER-targeted SMase and CDase showed quantitatively similar results to Golgi-targeted enzymes, but the increase in Sph was much more dramatic with the combination of SMase and CDase (Fig. 3C).

In contrast, the mitochondria-targeted CDase and SMase did not induce major changes in sphingolipid levels (Fig. 3D). Mitochondria-targeted bSMase increased Cer levels which was partially countered by mitochondria-targeted bCDase.
On the other hand, the nucleus-targeted CDase showed an increase of Sph level suggesting relative high levels of Cer in the nuclear membrane (Fig. 3E). Nucleus-targeted CDase slightly decreased the Cer formed by nucleus-targeted SMase, increasing Sph.

Finally, the expression of SMase in cytoplasm showed high access to SM pools (Fig. 3F). Moreover, the cytoplasmically-targeted CDase reduced this Cer and increased Sph (Fig. 3F).

Taken together, these results demonstrate biochemical cellular activities of the various targeted enzymes, with quantitative and qualitative differences in the sphingolipid composition of the different membranes.

**Analysis of intracellular ceramide molecular species modulated by organelle-targeted SMases and CDases**

The above results evaluated the total mass of Cer. However, it is becoming increasingly appreciated that ‘ceramide’ is indeed a family of structurally related molecules that demonstrate variations especially in the N-linked acyl chains. Therefore, we analyzed Cer molecular species in HeLa cells expressing the organelle-targeted SMase and CDase. For this, a heat map was developed in order to visualize the results. As shown in Fig. 4A, the results revealed that most of SMases induced significant increases of Cer species but with notable qualitative and quantitative differences depending on the targeted membrane. iPM-targeted SMase mainly acted on long chain SMs (C14-C18) but not on very long SMs (C22-C24), except for the unsaturated ones, which clearly behaved differently (C22:1-SM). Nucleus- and mitochondria-targeted SMases showed increases in the long chain Cers from C14 to C22, and C24:1-SM. ER-, Golgi- and cytoplasm-targeted SMases induced both long and very long chain Cers from C14 to C26. Interestingly, clustering of the molecular species revealed a distinct gradient of responses such that as targeted SMase ‘progressed’ from ER to Golgi to the entire cytoplasm, there was an accompanying increase in the levels of the longer chain Cers.

Consistent with the small/negligible effects of organelle-targeted CDase on total Cer, the various CDases had little effect on changes of Cer molecular species (Fig. 4B). As we previously predicted,
we did not expect Cer at the plasma membrane (26, 41), and this appears to be the case also for other membranes. Notably, especially for ER and Golgi, Cer levels increased in response to expressing CDase.

These two tools (SMase and CDase), informed us on what species of SM and Cer are present in different compartments. We also wanted to demonstrate if we can control the levels of Cer by co-expressing the SMase and the CDase in the same compartment. Supplemental figure 1 represents the remaining Cer in cells co-transfected with SMase+CDase. Here, our goal was to determine if the Cer generated by SMase was hydrolyzed when CDase was co-expressed in the same compartment. In order to better understand the effect of the CDase on the newly generated Cer, we plotted the difference of SMase and the co-expression of SMase+CDase (supplemental figure 1). Fig. 4C shows this difference (SMase – [SMase+CDase]). Inner PM-targeted SMase with CDase decreased the Cer levels from C14 to C16. Nucleus- and mitochondria-targeted SMases and CDase caused a significant decrease of Cers from C14 to C22. Furthermore, ER-, Golgi- and cytoplasm-targeted SMases and CDases reduced Cers from C14 to C24 and tended to prefer to metabolize very long chain Cers compared to long chain Cer when compared to other membranes. Therefore, it was found that Cer species produced by SMase and metabolized by CDase were different depending on each organelle they were targeted to.
DISCUSSION

This study describes the development of important tools that allow probing of Cer metabolism in distinct subcellular compartments; both the generation of Cer from SM and the clearance of Cer by ceramidases. The results reveal important differences in the molecular species of Cer-generated from SMases in various intracellular compartments. They also raise interesting considerations pertaining to sphingolipid metabolism. It is becoming increasingly clear that Cer is a family of closely related molecules that predominantly vary in the chain length of the acyl group as well as in various desaturations and hydroxylations. Importantly, these structural variations are the result of action of specific enzymes (hydroxylases, desaturases, as well as the 6 ceramide synthases that ‘specialize’ in introducing specific fatty acyl groups into Cer. These considerations are further compounded by the distinct subcellular localization of the various SMases, ceramidases and other Cer-metabolizing enzymes. Taken together, these prompted the formulation of the ‘Many Ceramide’ hypothesis whereby the biologic activities of Cer depend on the specific compartment where the specific Cer is present, which in turn is controlled by the location and regulation of those enzymes.

In order to understand how sphingolipids can be modulated by their involved enzymes in each organelle, we developed a set of tools of specific organelle-targeted bSMases and bCDases. These constructs showed active enzymes that were expressed in the intended compartment. Thus, they are useful for the ‘steady state’ reprogramming for compartment specific metabolism of SM and Cer. One limitation that would require future development is the need to develop acutely regulated and compartmentally-targeted enzymes. One important conclusion revealed by the results is that most targeted CDases did not increase hydrolysis of Cers by themselves. On the other hand, the combination of CDases with SMases revealed the ability of CDases to metabolize SMases-derived Cers. The reasons for lack of effects of CDases on their own are obscure at the moment. This may suggest that perhaps there are steady state reactive changes in Cer formation that counter any enhanced clearance of Cer. However, we did not observe changes in the molecular species of Cers with the action of bCDases. Moreover, CDases where capable of reducing the Cers generated from
SMases, which may suggest that, under these conditions, usual compensatory mechanisms are overwhelmed. One specific possibility is that endogenous ceramidases (which are compartmentally localized) are capable of dealing with steady state levels of Cer, and they become limiting only when Cer formation is driven by the action of SMases. These issues need further probing. The only notable exception was nucleus-targeted CDase which did increase levels of Sph but did not reduce Cer. The lack of change in Cer is consistent with the fact that Cer levels are many fold higher (approx. 30-40) than those of Sph so small changes in Cer are sufficient to generate significant differences in Sph. These results do disclose that the nucleus harbors Cers accessible to CDase action and that activation or induction of nuclear ceramidases may be sufficient to generate Sph. In the nucleus, SM, which is the most abundant nuclear sphingolipid, has been proposed to play both structural and regulatory roles in chromatin assembly and dynamics (39). It has also been reported that S1P modulates histone acetylation, such that S1P generated in the nucleus by SPHK2 regulates HDAC activity either by direct binding or through activation of nuclear reactive oxygen species and then modulates cell cycle and pro-inflammatory gene expression (42). On the other hand, Sph may serve as a ligand for steroidogenic factor 1. This current study shows that the nucleus-targeted SMase induces Cer levels and nucleus-targeted CDase is also tended to increase the cellular Sph level. At the very least then the nucleus-targeted SMase and CDase can emerge as very useful tools to probe specifically that compartment.

Next, the results showed that the chain length of the acyl group increased upon SMase expression as one proceeds from the ER to the Golgi to the entire cytoplasmic surfaces. Thus, ER showed a primary increase in C14-18 Cers, and Golgi showed a similar profile but with a tendency for the higher end of Cers in this range. On the other hand, cytoplasmically-targeted SMase increased Cer significantly, and it produced long chain Cer from C24 to C26 selectively. This SMase may have access to specific pools of SM with very long chain. The nature of these compartments where not defined in our study, but they would be interesting as they would suggest cytoplasmically-fac -
pertains to mitochondria. It has been shown that mitochondrial Cer levels are elevated prior to induction of apoptosis by CD95-, TNFα-radiation, or UV-induced and mitochondria are also capable of generating Cer via the reverse action of neutral ceramidase (23, 24). Here it is shown that mitochondria-targeted CDase hydrolyzes Cer from C14 to C24 produced by mitochondria-targeted SMase, suggesting presence of specific SMs in the mitochondria (43). The current results also bear on presence and metabolism of SM and Cer at the inner leaflet of the PM. nSMase2 has been known to be translocated to the plasma membrane in confluent cells (44), thus it is suggested that iPM-targeted SMase would be a useful tool for understanding nSMase2 functions. In this study, targeted SMase to the iPM generates Cers which are hydrolyzed into Sph by iPM-targeted CDase. This observation implies that stimuli such as hypoxia or ionizing radiation induce the activation of SMase to produce Cers in PM (45, 46).

It was also curious that the cytoplasmically-targeted bSMase increased ceramide levels substantially, suggesting that a significant amount of SM (perhaps up to 30% as suggested in Fig. 3) faces cytosolically. This is consistent with some previous studies on the ‘signaling’ pool of SM (47) and on finding SM in the inner leaflet of the PM (48).

In summary, we have developed tools to probe compartmentalized metabolism of SM and Cer through the use of organelle-targeted bSMases and bCDases in this study. The results reveal that SMase-derived Cer species are different in each organelle. These findings and tools can lead to better understanding of sphingolipid functions and physiological mechanism in a compartment-specific manner.

Acknowledgements.

We would like to thank Dr. Michael V. Airola and Ms. Prajna Shanbhogue for advice on experiments, and we also thank Toshiyuki Tabuchi, Isao Sugimoto and Kosuke Tani for helpful discussions. We thank the Lipidomics Core facility (a component of the Biological Mass Spectrometry Shared Resource) of the Stony Brook University Cancer Center for the lipid
analysis. We thank Dr. Antonio Delgado and Dr. Gemma Fabrias for compound RBM 14-16.

This work was supported in part by NIH grant CA97132 to YAH and also supported by Veteran’s Administration Merit Award (LMO) and NIGMS 9R01GM097741 (LMO).
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Table 1. Design of bSMase/bCDase constructs with targeting signal to different cellular compartments.

The inner PM-targeted signal is from c-Ha-ras, the mitochondrial-targeting sequence is isolated from subunit VIII of human cytochrome c oxidase, the nucleus-targeted sequence is isolated from the SV40 large T antigen, the ER-targeted sequence is from a mouse Vh chain, and the Golgi apparatus-targeted signal is from the human β1,4-galactosyltransferase.
Figure 1. Measurement of SMase and CDase activities in HeLa cells transfected with vectors containing compartmentally targeted enzymes.

Cells in which SMase and CDase genes were expressed were homogenated and incubated with substrate in reaction buffer. **A.** SMase *in vitro* activity. **B.** CDase *in vitro* activity. **C.** Expression of bSMases and bCDases in transfected HeLa cells. Results are representative of three experiments, ***p<0.0005, **p<0.005, *p<0.05.
Figure 2. Localization of targeted SMases and CDases tagged with V5 or Flag.

Cells were fixed and stained with anti-V5 or anti-Flag antibody (green), organelle markers (red, referred to in MATERIALS AND METHODS) and DAPI (blue), they were then observed by confocal microscopy. **A.** Cells transfected with SMase vectors containing targeting signal sequences. **B.** Cells transfected with targeted CDases. Results are representative of multiple experiments (>5).
Figure 3. Changes of SLs in HeLa cells transfected with compartmentally targeted CDases and SMases.

CDases and SMases containing PM (A), Golgi (B), ER (C), mitochondria (D), nucleus (E) and cytoplasm (F)-targeted sequences were used. HeLa cells were transfected with CDases or SMases and co-transfected with them and incubated for 20 hr. Cells were harvested, lipids extracted, and then subjected to HPLC-ES-MS analysis for quantification of the levels of sphingosine (Sph), sphingosine-1-phosphate (S1P), total ceramide (Cer) and total sphingomyelin.
(SM) normalized to lipid phosphate. Results are representative of three experiments,

***p<0.0005, **p<0.005, *p<0.05.
Figure 4. Heat diagram of profiles of ceramide molecular species in HeLa cells transfected with the organelle-targeted SMases and CDases.
The clustermap illustrates comprehensive quantification of ceramide species in cells overexpressed with SMase and CDase vectors containing the targeted signal sequence. All values are expressed as natural logarithmic-ratios and are color-coded from light green (decrease on ceramide) to red (increase on ceramide). **A.** Changes in ceramide levels from cells expressing respective SMase constructs (see X-axis). **B.** Changes in ceramide levels from cells expressing CDase constructs. **C.** Amounts of hydrolyzed ceramide by CDase constructs calculated as ceramide levels from cells expressing SMase constructs (which are generating ceramide, as shown in previous panel A) minus the remaining ceramide after co-expressing SMase and CDase. Please notice A and B are in logarithmic scale, where C is in linear scale.