Both Sphingomyelin Synthases SMS1 and SMS2 Are Required for Sphingomyelin Homeostasis and Growth in Human HeLa Cells*[S]

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Sphingomyelin (SM) is a vital component of cellular membranes in organisms ranging from mammals to protozoa. Its production involves the transfer of phosphocholine from phosphatidylcholine to ceramide, yielding diacylglycerol in the process. The mammalian genome encodes two known SM synthase (SMS) isoforms, SMS1 and SMS2. However, the relative contributions of these enzymes to SM production in mammalian cells remained to be established. Here we show that SMS1 and SMS2 are co-expressed in a variety of cell types and function as the key Golgi- and plasma membrane-associated SM synthases in human cervical carcinoma HeLa cells, respectively. RNA interference-mediated depletion of either SMS1 or SMS2 caused a substantial decrease in SM production levels, an accumulation of ceramides, and a block in cell growth. Although SMS-depleted cells displayed a reduced SM content, external addition of SM did not restore growth. These results indicate that the biological role of SM synthases goes beyond formation of SM.

Sphingolipids are ubiquitous components of biomembranes in eukaryotic cells. The most abundant sphingolipid species in mammalian cells is sphingomyelin (SM),[3] which comprises 5–15% of total phospholipids. SM synthesis involves the transfer of a phosphocholine head group from phosphatidylcholine (PC) to ceramide with concomitant production of diacylglycerol (DAG) (1, 2). The enzyme catalyzing this reaction, SM synthase, thus occupies a central position at a crossroad of sphingolipid and glycerolipid metabolism and has considerable biological potential as a regulator of the proapoptotic factor ceramide and mitogenic factor DAG.

Indeed several lines of evidence indicate that SM synthesis plays a critical role in cell growth and survival. Chinese hamster ovary mutant cells with a thermolabile serine palmitoyltransferase, the rate-limiting enzyme in sphingolipid synthesis, die in the absence of externally added sphingoid base when shifted to the non-permissive temperature. The mutant cells could be rescued by added SM but not by glucosylceramide (GlcCer), the precursor of higher glycosphingolipids (3). Another study reported the isolation of a mouse lymphoid cell line with diminished SM synthase activity and a defect in cell growth when cultured under serum-free conditions; growth could be restored by supplementing the medium with exogenous SM (4). Finally fluctuations in SM synthase activity have been linked to mitogenic and proapoptotic signaling in a variety of mammalian cell types (5–7).

The molecular basis for the requirement of SM synthesis in cell growth is not clear, and any of the following scenarios may apply. To begin with, SM accumulates in the outer leaflet of the plasma membrane, and its high packing density and affinity for cholesterol likely contribute to the barrier function of this organelle. In addition, SM at the plasma membrane provides a reservoir of lipid signaling molecules that are liberated by acidic or neutral SMases in response to a variety of biological stimuli (8); these molecules include ceramide, sphingosine, and sphingosine 1-phosphate, and have been implicated in the regulation of cell proliferation, differentiation, and apoptosis (9, 10). As SM has a strong capacity to form microdomains, its production in the *trans* Golgi may affect the lateral organization of other membrane components and hence provide a physical basis for sorting events that help establish the compositional and functional differences between the endoplasmic reticulum, plasma membrane, and Golgi itself (11). Moreover SM synthesis in the *trans* Golgi may create a local pool of DAG, which provides a cue for protein kinase D recruitment and the formation of secretory vesicles (12). Finally by regulating the cellular levels of the proapoptotic factor ceramide and mitogenic factor DAG in opposite directions, SM synthesis may have a direct impact on cell proliferation and life span (13, 14).

Using an expression cloning strategy in yeast, we previously identified a family of SM synthases whose members are present throughout the animal kingdom from mammals and nematodes to protozoa like the malaria parasite *Plasmodium falcip-*

[3] The abbreviations used are: SM, sphingomyelin; DAG, diacylglycerol; GlcCer, glucosylceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SMS, sphingomyelin synthase; siRNA, small interfering RNA duplex; NBD, 12-(*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); NS, non-silencing; LA, lamin A; HBSS, Hanks' buffered saline solution; BSA, bovine serum albumin; EPC, ethanolamine phosphorylceramide; MS, mass spectrometry.

arum (15, 16). In fact, each organism capable of SM production displays a multiplicity of SM synthase (SMS) genes. The human genome contains two of these genes, named SMS1 and SMS2. The corresponding enzymes fulfill essentially all criteria attributed previously to SM synthase and reside in organelles where SM synthesis is known to occur, namely the Golgi (SMS1) and plasma membrane (SMS2) (15, 17). In line with these findings, Yamaoka *et al.* (4) reported the expression cloning of human SMS1 using a mouse lymphoid cell line with severely reduced SM synthase activity and susceptibility to methyl-β-cyclodextrin-induced cell death. This work suggested that SMS1 represents a major SM synthase activity in mammalian cells with a critical role in cell growth. However, the molecular basis of the SM synthase defect in the lymphoid cell line has not yet been clarified. Interestingly a recent study showed that resistance of S49 mouse lymphoma cells to alkyl-lysophospholipid-induced apoptosis is due to transcriptional down-regulation of SMS1 (18). Whether alkyl-lysophospholipid-resistant S49 cells are defective in growth when cultured under serum-free conditions (*i.e.* without exogenous SM) is unclear. Consequently the precise contribution of SMS1 and SMS2 to SM production and growth in mammalian cells remains to be determined.

In the present study we used RNA interference to specifically deplete SMS1 or SMS2 from human cervical carcinoma HeLa cells and analyzed the effect of these manipulations on the Golgi- and plasma membrane-associated SM synthase activities, SM production levels, overall lipid composition, and cell growth.

EXPERIMENTAL PROCEDURES

Chemicals—NBD-hexanoylceramide (C_6-NBD-ceramide) was from Molecular Probes (Eugene, OR). Percoll™ was from Amersham Biosciences. *N*-Methyl[^{14}C]choline, 3-L-[^{14}C]serine, and 1-[^{3}H]sphingosine (specific activity, 30–60 mCi/mmol) were from MP Biomedicals (Irvine, CA). All other chemicals were from Sigma-Aldrich.

Cell Culture, Plasmid Transfection, and RNA Interference—HeLa cells were routinely cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were transfected with human SMS1-V5/pcDNA3.1, human SMS2-V5/pcDNA3.1 (15), or mouse α-mannosidase II-RFP/plk13 (a gift from L. Krueschner, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) using Lipofectamine 2000 (Invitrogen). Stable transfectants were selected in medium containing 0.8 mg/ml G418 (Invitrogen). RNA interference was performed on HeLa cells transfected with small interfering RNA duplexes (siRNAs; Qiagen, Germantown, MD) using Oligofectamine (Invitrogen) as described previously (19). All transfections with siRNAs were carried out in Opti-MEM I Glutamax medium (Invitrogen) supplemented with 3% normal or delipidated fetal calf serum as indicated. Delipidated serum was prepared by butanol-diisopropyl ether extraction according to Cham and Knowles (20). Several different SMS1- and SMS2-directed siRNAs were initially tested on SMS1-V5- and SMS2-V5-expressing HeLa cells. This yielded one SMS1 siRNA (sense, CUACA-CUCCCAGUACCUGG) and three different SMS2 siRNAs (2a sense, GGCUCAAUUCCUUGCUGCU; 2b sense, CCCAA-

GAGCUUAUCCAGUG; 2c sense, GUCAAUAGUGGGACG-CAGA) that each gave a strong (80–90%) and specific reduction in expression of the corresponding SMS protein after 3 days of treatment. Non-silencing (NS) siRNA (sense, UUCUC-CGAACGUGUCACGU) and siRNA directed against the nuclear matrix protein lamin A (LA; sense, CUGGACUUCCA-GAAGAACA) served as controls. For long term RNA interference, cells were passaged after 3 days of siRNA transfection, retransfected with siRNAs, and then incubated for 3 or 4 additional days as indicated.

Antibodies, Immunoblotting, and Immunofluorescence Microscopy—HeLa cells and yeast strains expressing V5-tagged SMS1 or SMS2 were processed for immunoblot analysis as described previously (15). An 18-amino acid peptide (CRKYS-RVQKIGEDNEKST) corresponding to the C terminus of human SMS2 was coupled to keyhole limpet hemocyanin and used to immunize rabbits (Exalpha Biologicals Inc., Maynard, MA). One of the resulting antisera (antiserum 15910) was selected for this study and used at a dilution of 1:1,000 for immunoblot analysis. Rabbit polyclonal anti-rat Na^+/K^+-ATPase antibody (M09-C356) was a gift from J. Koendering (Centre for Molecular Life Sciences, Radboud University Nijmegen). Rabbit polyclonal anti-lamin A/C antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal anti-GM130 antibody was from BD Biosciences. Antibodies against the V5 epitope and p24 protein were described previously (15). For immunoblot analysis, antibody incubations were carried out in phosphate-buffered saline containing 2% ovalbumin and 0.5% Tween 20. After incubation with peroxidase-conjugated secondary antibodies (Bio-Rad), blots were developed using a chemiluminescent substrate kit (Pierce). Chemiluminescent bands were quantified using a GS-710 calibrating imaging densitometer (Bio-Rad) with QuantityOne software. Immunofluorescence microscopy was carried out as described previously (15).

Cell Fractionation and Enzyme Assays—Subcellular fractionations on Percoll density gradients were performed as described previously (21) with minor modifications. Briefly HeLa cells grown on 15-cm dishes were scraped and disrupted in ice-cold lysis buffer (10 mM Tris/HCl, pH 7.4, 0.25 M sucrose containing freshly added protease inhibitors) (15) with 80 strokes of a loose fitting Dounce homogenizer. Homogenates were centrifuged at $700 \times g$ for 15 min, and pellets were washed once in ice-cold lysis buffer. Supernatants were pooled, and Percoll was added to a final concentration of 22.5% (v/v). Lysates were centrifuged at 17,000 rpm in a Beckman 50Ti rotor for 30 min at 4 °C, and 24 0.4-ml fractions were collected from the top. Equal amounts per fraction were subjected to immunoblotting and analyzed for GlcCer and SM synthase activity. To this end, gradient fractions (100 μl) were incubated for 1 h at 37 °C in Hanks' buffered saline solution (HBSS) containing 10 μM C_6-NBD-Cer, 1 mM UDP-glucose, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ in a total volume of 0.5 ml. Lipids were extracted by addition of 1.7 ml of chloroform:methanol (1:2.2), dried under N_2, and subjected to butanol/water partitioning. Lipids recovered from the butanol phase were separated by TLC for 30 min in chloroform:acetone:methanol:acetic acid: water (50:20:10:10:5, v/v/v/v/v). Fluorescent lipids were visual-

ized on a Storm 860 imaging analysis system (GE Healthcare) and quantified with ImageQuant software.

Metabolic Labeling—HeLa cells were labeled with *N*-methyl[^{14}C]choline (2 μCi/ml) or 3-L-[^{14}C]serine (1 μCi/ml) for 4 h or in the case of 1-[^{3}H]sphingosine (1 μCi/ml) for 1 h in Opti-MEM containing 3% delipidated serum at 37 °C. Cells were washed in phosphate-buffered saline and then subjected to lipid extraction using the method of Bligh and Dyer (22). The organic extracts were dried under N$_2$ and, where indicated, deacylated by mild base treatment in 0.5 M methanolic NaOCH$_3$ (60 min at room temperature). Lipids were extracted with chloroform and separated by TLC in chloroform, methanol, 25% ammonia solution (50:25:6, v/v/v) or in the case of 1-[^{3}H]sphingosine labeling using chloroform, methanol, 0.2% CaCl$_2$ (60:40:9, v/v/v). Radiolabeled lipids were detected by exposure to BAS-MS imaging screens (Fuji Photo Film Co.), read out on a Bio-Rad Personal Molecular Imager, and quantified with ImageQuant software.

Mass Spectrometric Analysis—HeLa cell lipid extracts were prepared according to the method of Folch *et al.* (23) but omitting the salt. Crude lipid extracts were evaporated under N$_2$, dissolved in chloroform:methanol (1:2), and supplemented with internal standards for SM, PC, phosphatidylethanolamine (PE), phosphatidylserine, phosphatidylinositol, ceramide, and GlcCer as described previously (24). Phospholipid concentration was determined by phosphate analysis (25), sphingolipid concentration was determined according to Naoi *et al.* (26), and cholesterol concentration was determined as described in Gamble *et al.* (27). The lipid molecular species and classes were quantified by liquid chromatography-MS as detailed in Hermansson *et al.* (24) using an Interchrom Lichrosphere diol-modified silica column coupled on line to a Quattro Micro triple quadrupole mass spectrometer (Micromass, Manchester, UK).

C$_6$-NBD-ceramide Staining of the Golgi—C$_6$-NBD-ceramide labeling of live HeLa cells was performed as described previously (28) with some minor modifications. Briefly subconfluent cells grown on glass coverslips were incubated for 30 min in HBSS containing 5 μM C$_6$-NBD-ceramide complexed with 5 μM fatty acid-free BSA at 4 °C. Cells were washed three times, chased in HBSS + 1% BSA for 10 min at 37 °C, and then fixed with 0.125% glutaraldehyde in HBSS for 5 min at 4 °C. Cells were immediately observed using a Nikon D-Eclipse C1 microscope.

Cell Surface SM Synthase Assay—Cell surface SM synthase activity was assayed essentially as described previously (29). In brief, subconfluent HeLa cells were washed with ice-cold HBSS and incubated for 3 h at 0 °C in HBSS containing 5 μM C$_6$-NBD-ceramide and 1% (w/v) fatty acid-free BSA followed by a 30-min incubation in HBSS + 1% (w/v) BSA. The medium was pooled, and lipids were extracted by the method of Bligh and Dyer (22) and subjected to butanol/water partitioning. Lipids recovered from the butanol phase were separated by one-dimensional TLC in chloroform, methanol, 25% ammonia solution (9:7:3, v/v/v). Fluorescent lipids were visualized and quantified as above.

Cell Growth Assay—HeLa cells plated at 2 × 10^5 cells/9.6-cm^2 dish were treated with siRNAs in Opti-MEM as above.
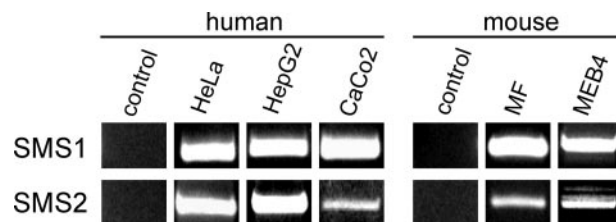

FIGURE 1. **SMS1 and SMS2 are co-expressed in various mammalian cell types.** Reverse transcription-PCR of SMS1 and SMS2 mRNAs was carried out on total RNA (~0.5 μg/reaction) isolated from human cervical carcinoma HeLa cells, human hepatoma HepG2 cells, human colon carcinoma CaCo2 cells, mouse fibroblast MF cells, and mouse melanoma MEB4 cells using the Titan One Tube reverse transcription-PCR system (Roche Applied Science). To verify that the SMS1- and SMS2-specific primers themselves did not result in a product, RNA was omitted in control reactions.

After 6 h of treatment, the medium was changed for Opti-MEM containing 3% normal or 3% delipidated fetal calf serum supplemented with 80 μM exogenous SM or PC as described previously (3). In brief, lipids were dissolved in a small amount of chloroform, dried under sterile conditions, and then dispersed in autoclaved water at concentrations of 5–10 mM with a bath type sonicator for 30 min at room temperature. At the indicated time points, cells were washed with phosphate-buffered saline, trypsinized, and resuspended in HBSS containing 1% fetal calf serum. Next 100 ng/ml propidium iodine was added, and the number of viable and dead cells was analyzed on a FACSCalibur flow cytometer using CellQuest software. To determine apoptotic events, cells were first resuspended in binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl$_2$, pH 7.4) and then labeled with fluorescein isothiocyanate-conjugated annexin V. After 15 min of incubation, 100 ng/ml propidium iodine was added, and the cells were analyzed by flow cytometry as above.

RESULTS

SMS1 and SMS2 Are Co-expressed in Various Cell Types— Northern blot analysis previously showed that SMS1 and SMS2 are expressed in all major human tissues (15). Therefore, one may anticipate that most cell types contain both SM synthase isoforms. To verify this, we analyzed various mammalian cell lines for SMS1 and SMS2 expression using reverse transcription-PCR. Total RNA was isolated from human cervical carcinoma HeLa cells, human hepatoma HepG2 cells, human colon carcinoma CaCo2 cells, mouse melanoma MEB4 cells, and mouse fibroblast MF cells. As shown in Fig. 1, reverse transcription-PCR revealed the presence of both SMS1 and SMS2 transcripts in all five cell lines analyzed. These results indicate that SMS1 and SMS2 are encoded by ubiquitously expressed genes.

Characterization of SMS1- and SMS2-overexpressing HeLa Cells—SMS1 and SMS2 reside in organelles where SM synthesis is known to occur, namely in the Golgi and at the plasma membrane, respectively (15). However, whether SMS1 and SMS2 are responsible for SM synthesis at these locations is not known. This led us to analyze the impact of SMS1 and SMS2 overexpression and depletion on Golgi- and plasma membrane-associated SM synthase activities. To this end, HeLa cells were stably transfected with V5 epitope-tagged SMS1 or SMS2 constructs. Consistent with our previous work (15), SMS1-V5 localized exclusively to the perinuclear region where it displayed extensive co-localization with Golgi marker GM130
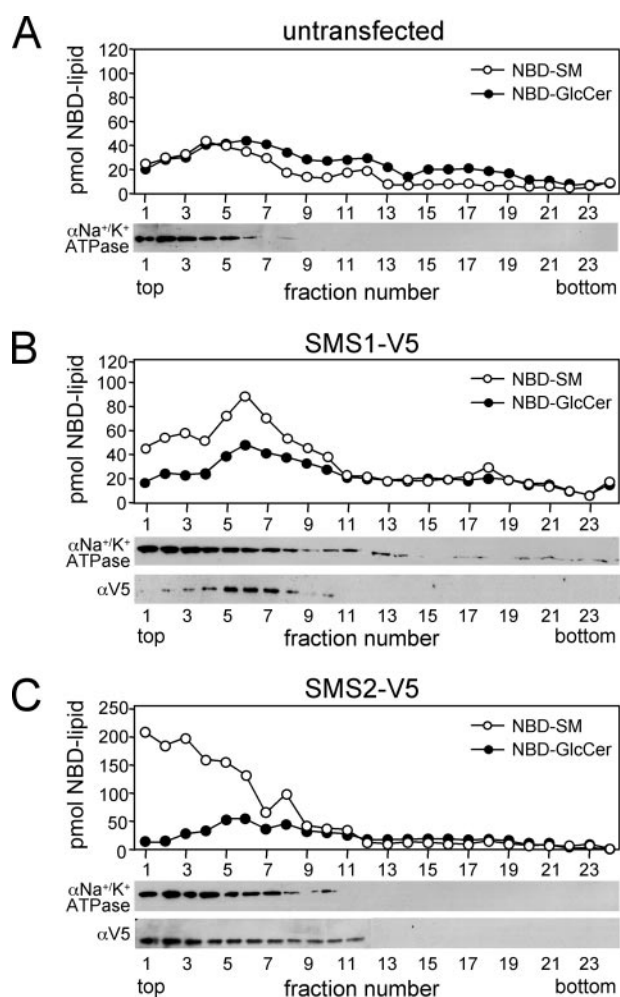
FIGURE 2. **Subcellular fractionation of SM synthase activity from SMS1- and SMS2-overexpressing HeLa cells.** Postnuclear supernatants of untransfected HeLa cells (*A*) or HeLa cells stably transfected with SMS1-V5 (*B*) or SMS2-V5 constructs (*C*) were fractionated on 22.5% Percoll gradients. Fractions were collected from the top and analyzed for SM synthase activity and GlcCer transferase activity as described under "Experimental Procedures." In brief, fractions were incubated with C_6-NBD-ceramide, and the products formed (NBD-SM and NBD-GlcCer) were separated by one-dimensional TLC and quantified using a fluorescence imager. In addition, fractions were immunoblotted with mouse monoclonal anti-V5 and rabbit polyclonal anti-plasma membrane Na^+/K^+-ATPase antibodies.

FIGURE 3. **siRNA-induced down-regulation of SMS1 and SMS2 expression in HeLa cells.** *A*, SMS1-V5- or SMS2-V5-expressing HeLa cells were treated with siRNAs targeting LA, SMS1, or SMS2 for 3 days and then processed for immunoblot analysis. Blots were stained with antibodies directed against the V5 epitope, LA, or p24 protein. *B*, immunoblots of membrane extracts from yeast cells transfected with empty vector (*control*) or with expression vectors containing SMS1-V5 or SMS2-V5. Blots were stained with anti-V5 antibody or a rabbit polyclonal antibody directed against the C terminus of SMS2. The positions of intact SMS2-V5 and a C-terminal SMS2-V5 degradation product are indicated with an *arrow* and an *arrowhead*, respectively. The position of a cross-reacting yeast protein is indicated by an *asterisk*. *C*, HeLa cells were treated with LA, SMS1, or SMS2 siRNAs for 3 days and then processed for immunoblot analysis. Blots were stained with rabbit polyclonal anti-SMS2 and anti-plasma membrane Na^+/K^+-ATPase antibodies.

(supplemental Fig. 1). SMS2-V5, on the other hand, primarily localized to the plasma membrane. A small portion of the protein was also found in the perinuclear region where it co-localized with GM130.

In line with the immunofluorescence microscopy data, subcellular fractionation on Percoll density gradients showed that SMS1-V5 co-migrated with the Golgi-resident GlcCer transferase activity and segregated from the bulk of Na^+/K^+-ATPase, a marker of the plasma membrane (Fig. 2A). In contrast, the fractionation profile of SMS2-V5 matched that of Na^+/K^+-ATPase and was distinct from the fractionation profile of GlcCer transferase (Fig. 2B). The SM synthase activity in SMS1-V5-expressing cells was 2-fold higher than in untransfected cells (891 *versus* 427 pmol of C_6-NBD-SM formed/mg of protein/h) and co-migrated with SMS1-V5 and GlcCer transferase (Fig. 2A). In SMS2-V5-expressing cells on the other hand, the SM synthase activity displayed a 4-fold increase rela-
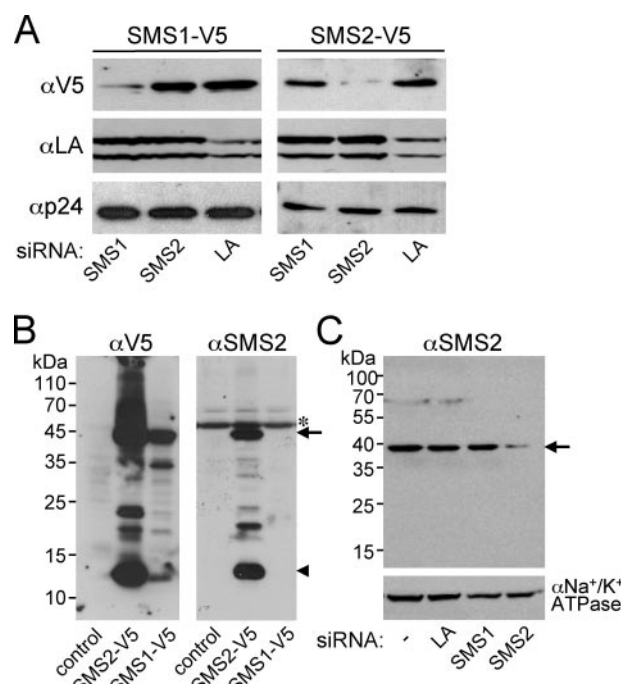
tive to untransfected cells (1,662 *versus* 427 pmol of C_6-NBD-SM formed/mg of protein/h) and co-fractionated with SMS2-V5 and Na^+/K^+-ATPase (Fig. 2B). Hence overexpression of SMS1 primarily increases the Golgi-associated SM synthase activity, whereas overexpression of SMS2 primarily increases the SM synthase activity at the plasma membrane.

SMS1 Represents a Major whereas SMS2 Represents a Minor SM Synthase Activity in HeLa Cells—To address whether SMS1 and SMS2 are responsible for the endogenous Golgi- and plasma membrane-associated SM synthase activities in HeLa cells, their expression was blocked using siRNAs. NS siRNA and siRNA directed against the nuclear matrix protein LA served as controls. Several different SMS1- and SMS2-directed siRNAs were initially tested on SMS1-V5- and SMS2-V5-expressing HeLa cells. This yielded one SMS1 siRNA and three different SMS2 siRNAs that each gave a strong (80–90%) and specific reduction in expression of the corresponding SMS protein after 3 days of treatment (Fig. 3A and data not shown). To verify that these siRNAs were also effective in down-regulating expression of endogenous SMS proteins, we raised a polyclonal antibody against an 18-amino acid synthetic peptide corresponding to the C terminus of human SMS2. This antibody cross-reacted with human SMS2 expressed in yeast (Fig. 3B) and primarily recognized a 38-kDa protein in HeLa membrane

extracts (Fig. 3*C*). The latter protein represents endogenous SMS2 because its expression in HeLa cells was drastically (~80%) reduced after treatment with SMS2 siRNA but unaffected after treatment with LA or SMS1 siRNAs (Fig. 3*C*).

To determine the individual contributions of SMS1 and SMS2 to the total cellular SM synthase activity, HeLa cells were treated for 3 or 7 days with LA, SMS1, or SMS2 siRNAs; lysed; and then incubated with C_6-NBD-ceramide in the presence of *N*-ethylmaleimide, an inhibitor of mammalian SMases that does not interfere with SM synthase activity (15). Formation of C_6-NBD-SM was monitored by TLC and then normalized for total amount of protein. As shown in Fig. 4*A*, cells treated for 3 days with SMS1 siRNA showed a major (~60%) reduction in SM synthase activity compared with LA siRNA-treated cells. Moreover these cells proved to be virtually devoid of the SM synthase activity peak that normally co-migrates with the GlcCer transferase peak on a Percoll density gradient (Fig. 4*B*). When the siRNA treatment was prolonged to 7 days, the SM synthase activity in SMS1-depleted cells dropped even further, namely to ~20% of that in LA-depleted cells (~80% reduction; Fig. 4*A*). SMS2-depleted cells, on the other hand, displayed a less pronounced reduction in SM synthase activity (~40% reduction after 7 days of siRNA treatment; Fig. 4*A*) and, unlike SMS1-depleted cells, still contained the bulk of SM synthase activity that co-migrates with GlcCer transferase when fractionated on a Percoll density gradient (Fig. 4*B*). When cells were treated simultaneously with SMS1 and SMS2 siRNAs for 7 days, the SM synthase activity was reduced to nearly 10% of that in LA-depleted cells (~90% reduction; Fig. 4*A*). These results indicate that SMS1 and SMS2 together make up the bulk of SM synthase activity in HeLa cells with SMS1 acting as a major SM synthase activity and SMS2 acting as a minor one.

SMS1 Depletion Abolishes C_6-NBD-ceramide Staining of the Golgi Complex—The fluorescent ceramide analogue C_6-NBD-ceramide stains the Golgi complex (30) and has been applied to study the dynamics of this organelle in living cells (31). The mechanism by which C_6-NBD-ceramide accumulates in the Golgi is unclear. Fluorescence microscopy of live yeast incubated with C_6-NBD-ceramide revealed bright cytoplasmic patches similar in appearance to the Golgi (32). Interestingly this staining was absent in cells treated with aureobasidin A, a potent inhibitor of the enzyme responsible for inositol phosphorylceramide synthesis in the Golgi lumen. We reasoned that, analogous to the situation in yeast, C_6-NBD-ceramide staining of the Golgi in mammalian cells might require an active SM synthase in the Golgi. To investigate this possibility, HeLa cells were treated with LA, SMS1, or SMS2 siRNA for 3 days and then stained with C_6-NBD-ceramide as described under "Experimental Procedures." Cells depleted of LA or SMS2 showed a bright perinuclear staining reminiscent of the Golgi complex (Fig. 5). However, this perinuclear staining was strongly reduced in a substantial portion (up to 50%) of SMS1-depleted cells. Instead these cells primarily showed a faint staining of the endoplasmic reticulum and nuclear envelope.

To investigate the possibility that the absence of perinuclear staining in SMS1-depleted cells was due to collapse of the Golgi complex, we next used HeLa cells stably expressing a red fluorescent protein-tagged version of the Golgi-resident enzyme
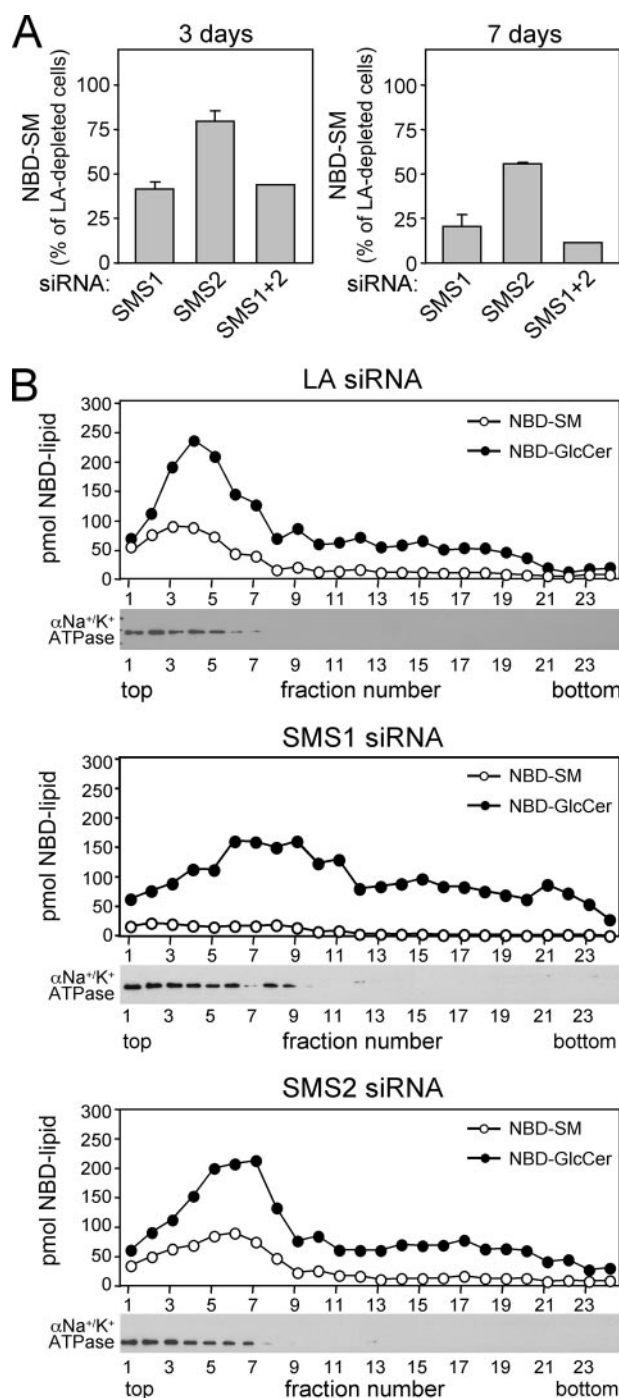

FIGURE 4. **Effect of SMS1 and SMS2 depletion on total SM synthase activity in HeLa cells.** *A*, HeLa cells were treated for 3 or 7 days with SMS1, SMS2, or both SMS1 and SMS2 siRNAs; then lysed; and incubated with C_6-NBD-ceramide. Formation of NBD-SM was monitored by TLC, normalized against total protein, and then expressed as percentage of the amount of NBD-SM formed in LA siRNA-treated cells. Data shown are means ± S.D. of three independent experiments (except for data on cells treated with SMS1 and SMS2 siRNAs simultaneously that were obtained from a single experiment). *B*, postnuclear supernatants derived from HeLa cells pretreated with LA, SMS1, or SMS2 siRNA for 3 days were fractionated on 22.5% Percoll gradients. Fractions were collected from the top, analyzed for SM synthase activity and GlcCer transferase activity, and immunoblotted with anti-plasma membrane Na^+/K^+-ATPase antibodies as described in the legend of Fig. 2.

α-mannosidase II. As shown in Fig. 6, the perinuclear C_6-NBD-ceramide staining in LA- or SMS2-depleted cells co-localized with red fluorescent protein-tagged α-mannosidase II. In con-
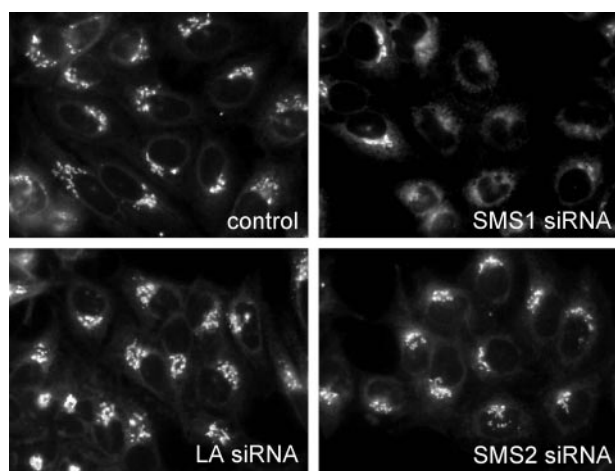
FIGURE 5. **SMS1 depletion abolishes C$_6$-NBD-ceramide staining of the Golgi complex.** HeLa cells were treated with LA, SMS1, or SMS2 siRNAs for 3 days and then incubated with C$_6$-NBD-ceramide for 30 min at 4 °C. Next cells were washed, chased for 10 min at 37 °C in BSA-containing medium, fixed, and visualized by fluorescence microscopy.

FIGURE 6. **SMS1 depletion has no general impact on the structural integrity of the Golgi complex.** HeLa cells expressing a red fluorescent protein-tagged version of the medial Golgi marker α-mannosidase II (*MannII-RFP*) were treated with LA, SMS1, or SMS2 siRNAs for 3 days and then incubated with C$_6$-NBD-ceramide for 30 min at 4 °C. Next cells were washed, chased for 10 min at 37 °C in BSA-containing medium, fixed, and visualized by fluorescence microscopy.
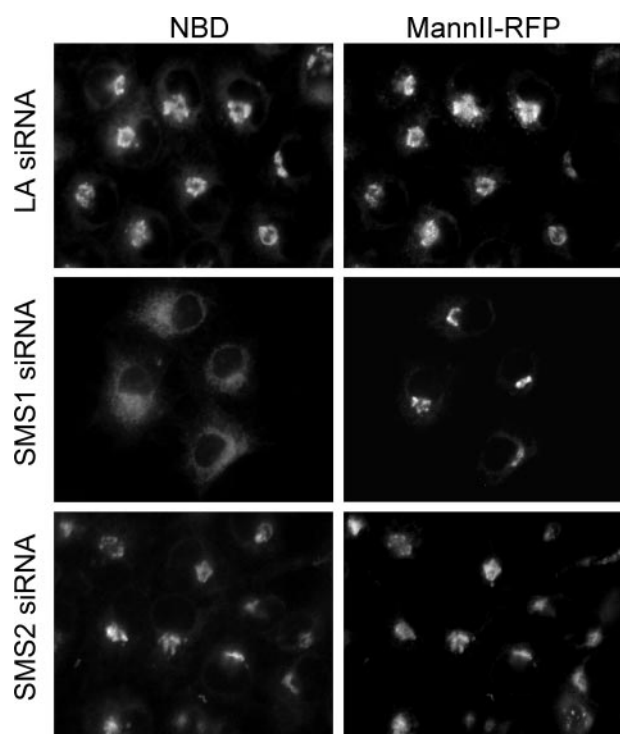
trast, although SMS1-depleted cells contained red fluorescent protein-labeled perinuclear structures reminiscent of the Golgi complex, these structures were devoid of C$_6$-NBD-ceramide staining. These findings suggest that C$_6$-NBD-ceramide labeling of the Golgi requires ongoing SM synthesis in the Golgi lumen. Moreover they provide complementary evidence that SMS1 is the key Golgi-associated SM synthase in HeLa cells.
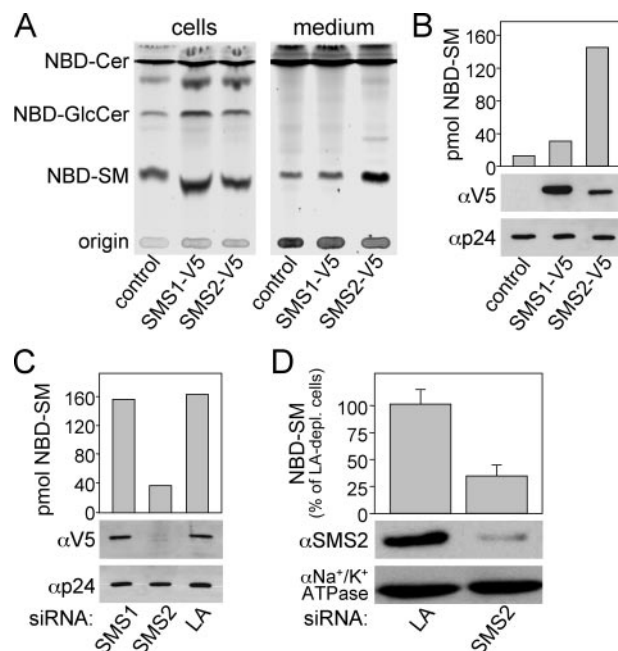

FIGURE 7. **SMS2 serves as the principal plasma membrane-associated SM synthase activity in HeLa cells.** *A*, normal HeLa cells (*control*) or HeLa cells expressing SMS1-V5 or SMS2-V5 were incubated for 3 h at 0 °C with 5 μM C6-NBD-ceramide in BSA-containing medium. NBD-SM synthesized on the cell surface was depleted by the BSA in the medium. NBD-labeled lipids were extracted from cells and medium, separated by TLC, and then visualized on a fluorescence imager. *B*, the amount of NBD-SM formed on the surface of normal, SMS1-V5-, or SMS2-V5-expressing HeLa cells was quantified using a fluorescence imager and after normalization against total cellular protein expressed in pmol. Cells were processed for immunoblotting with mouse monoclonal anti-V5 and rabbit polyclonal anti-p24 antibodies. *C*, HeLa cells expressing SMS2-V5 were treated with LA, SMS1, or SMS2 siRNAs for 3 days and then analyzed for cell surface-associated SM synthase activity as in *A*. *D*, normal HeLa cells were treated with LA or SMS2 siRNAs for 7 days and then analyzed for cell surface-associated SM synthase activity as in *A*. The amount of NBD-SM formed on the cell surface was normalized against total cellular protein and then expressed as the percentage of cell surface-derived NBD-SM in LA siRNA-treated cells. Data shown in *D* are means ± S.D. of three independent experiments. Cells were processed for immunoblotting with rabbit polyclonal anti-SMS2 and anti-plasma membrane Na$^+$/K$^+$-ATPase antibodies. *depl.*, depleted.

SMS2 Is the Principal Plasma Membrane-associated SM Synthase in HeLa Cells—Cell fractionation data on rat liver indicate that, although most SM synthase activity is associated with the Golgi, about 10% localizes to the plasma membrane (33). Cell surface SM synthase assays on dog epithelial Madin-Darby canine kidney cells confirmed that a minor fraction of the total SM synthase activity is associated with the plasma membrane (29). SMS2 predominantly resides at the plasma membrane and therefore is a strong candidate for the cell surface-associated SM synthase. To test whether SMS2 is responsible for this activity, HeLa cells overexpressing or deficient in SMS2 were analyzed for cell surface SM synthase activity using the assay developed by van Helvoort *et al.* (29). To this end, cells were incubated with C$_6$-NBD-ceramide for 3 h on ice in medium supplemented with BSA to extract any newly formed C$_6$-NBD-SM from the cell surface. Under these conditions, vesicular traffic is blocked so that C$_6$-NBD-SM produced in the Golgi is unable to reach the cell surface.

As shown in Fig. 7*A*, some newly synthesized C$_6$-NBD-SM could be extracted from normal HeLa cells. This was not due to leakage from damaged cells because newly synthesized

C_6-NBD-glucosylceramide, a product synthesized from C_6-NBD-ceramide on the cytosolic surface of the Golgi, could not be extracted from the cells. SMS2-V5-expressing cells displayed a 10-fold increase in the cell surface-associated SM synthase activity (Fig. 7, *A* and *B*). In contrast, cells expressing SMS1-V5 showed only a minor (2-fold) increase in this activity. Depletion of LA or SMS1 had no effect on the cell surface-associated SM synthase activity in SMS2-V5-expressing cells. However, this activity was strongly reduced upon depletion of SMS2 (Fig. 7*C*). Moreover normal HeLa cells depleted for SMS2 displayed a strong (~70%) reduction in cell surface-associated SM synthase activity compared with LA-depleted cells (Fig. 7*D*). Collectively these data indicate that SMS2 is primarily responsible for the cell surface-associated SM synthase activity in HeLa cells.

Both SMS1 and SMS2 Contribute to SM Production in HeLa Cells—To determine the contributions of SMS1 and SMS2 to SM synthesis *in vivo*, siRNA-treated HeLa cells were metabolically labeled for 4 h with the SM precursor *N*-methyl[^{14}C]choline, and the amount of radiolabeled SM formed was quantified by TLC and autoradiography. To exclude uptake of substantial amounts of exogenous SM from the medium (which may influence endogenous production levels) while avoiding the harsh conditions associated with serum deprivation, siRNA treatment and metabolic labeling was performed in Opti-MEM containing 3% delipidated serum. Remarkably cells treated with SMS1 or SMS2 siRNAs for 3 days displayed a 4- and 2-fold decrease in [^{14}C]PC levels compared with NS or LA siRNA-treated cells, respectively.[4] This was likely due to a general down-regulation of phospholipid biosynthesis in SMS-depleted cells because incorporation of 3-L-[^{14}C]serine into phosphatidylserine was affected to a similar extent (3- and 2-fold decrease in SMS1 and SMS2 siRNA-treated cells, respectively). Nevertheless after normalization against [^{14}C]PC levels, SMS1-depleted cells displayed a marked reduction in the amount of [^{14}C]SM compared with NS or LA siRNA-treated cells, namely from 45% after 3 days to 70% after 7 days of depletion (Fig. 8*A* and data not shown). In comparison, SMS2-depleted cells displayed a less pronounced but still significant decrease in [^{14}C]SM levels, namely from 20% after 3 days to 40% after 7 days of depletion. Very similar reductions in SM production levels were observed when SMS-depleted cells were labeled with two alternative SM precursors, 3-L-[^{14}C]serine and 1-[^{3}H]sphingosine (Fig. 8, *A* and *B*). These results demonstrate that both SMS1 and SMS2 contribute to SM production in HeLa cells.

Impact of SMS1 and SMS2 Depletion on the Lipid Composition of HeLa Cells—We next determined the effect of SMS1 and SMS2 depletion on the overall lipid composition of HeLa cells using liquid chromatography and mass spectrometric analysis. To exclude uptake of substantial amounts of lipid from the culture medium, siRNA treatment was performed on cells cultured in Opti-MEM containing 3% delipidated serum. Remarkably cells treated with SMS1 siRNA for 7 days displayed only a
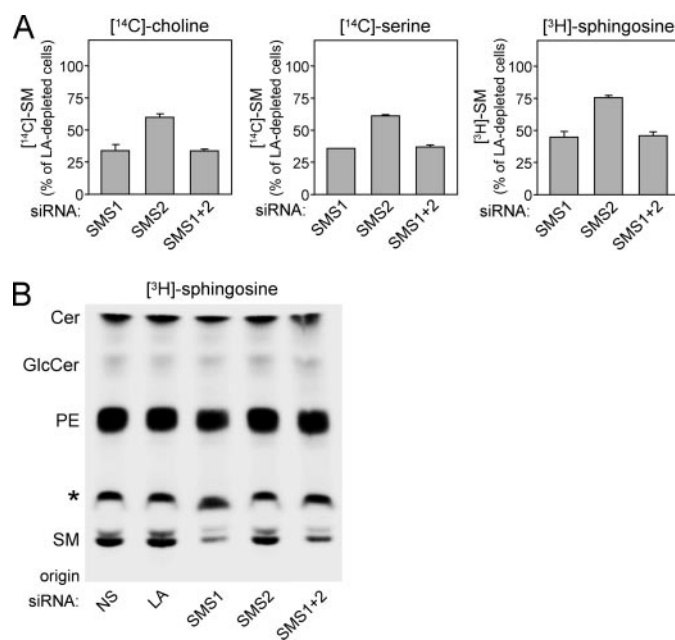

FIGURE 8. **SMS1 and SMS2 each contribute to SM production in HeLa cells.** *A*, HeLa cells treated with SMS1, SMS2, or SMS1 and SMS2 siRNAs for 7 days were metabolically labeled with methyl[^{14}C]choline or 3-L-[^{14}C]serine for 4 h or with 1-[^{3}H]sphingosine for 1 h at 37 °C. Formation of radiolabeled SM was monitored by TLC; normalized against [^{14}C]choline-labeled PC, [^{14}C]serine-labeled phosphatidylserine, or ^{3}H-labeled PE, respectively; and then expressed as the percentage of radiolabeled SM formed in LA siRNA-treated cells. Data shown are means ± S.D. of two independent experiments. *B*, lipids extracted from siRNA-treated, 1-[^{3}H]sphingosine-labeled HeLa cells (as in *A*) were separated by one-dimensional TLC and then visualized by autoradiography. The location of SM, ceramide (*Cer*), GlcCer, PE (PE is probably labeled via degradation of sphingosine 1-phosphate to phosphoethanolamine), and a lipid of unknown identity (*asterisk*) is indicated.

minor (up to 20%) reduction in SM levels compared with NS or LA siRNA-treated cells, namely from 7.7 to 6.3 mol % of total phospholipid (Table 1). This reduction was reached within 3 days of siRNA treatment[4] and affected all major SM species to a similar extent (Fig. 9). Moreover it was accompanied by a 1.8-fold increase in ceramide and a 3-fold increase in GlcCer levels. Compared with SMS1 siRNA-treated cells, cells treated with SMS2 siRNAs showed a slightly less pronounced reduction in SM levels (maximum, 14%), a similar (1.7-fold) increase in ceramide levels, and only a very minor (1.3-fold) increase in GlcCer levels (Table 1 and Fig. 9). The molecular species composition of the ceramides that accumulate in SMS1- and SMS2-depleted cells was virtually identical, indicating that SMS1 and SMS2 have common substrate specificity. Cells treated with both SMS1 and SMS2 siRNAs displayed a lipid composition very similar to that of SMS1-depleted cells. SMS depletion had no significant effect on the proportion of the major phosphoglycerolipid classes (PC, phosphatidylserine, PE, and phosphatidylinositol) or on the levels of cholesterol or DAG (Table 1).[4]

The limited impact of SMS depletion on cellular SM levels was rather unexpected in light of the strongly reduced enzyme activity (80–90%; Fig. 4*A*). However, cells treated with SMS siRNAs under serum-free conditions also showed only a modest (maximum, 20%) reduction in SM levels.[4] Hence the limited impact of SMS depletion on cellular SM levels cannot be ascribed to uptake of residual SM from delipidated serum.

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[4] F. Geta Tafesse, K. Huitema, M. Hermansson, S. van der Poel, J. van den Dikkenberg, A. Uphoff, P. Somerharju, and J. C. M. Holthuis, unpublished results.

TABLE 1

Lipid composition of control and SMS-depleted HeLa cells

HeLa cells cultured in Opti-MEM containing 3% delipidated serum were treated for 7 days with NS, LA, SMS1, and/or SMS2 siRNA, then subjected to lipid extraction, and analyzed by liquid chromatography-MS as described under "Experimental Procedures." Levels of sphingomyelins, ceramides, glucosylceramides, and phosphatidylcholines are given as mol % and relative to levels in cells treated with NS siRNA. Data shown are means ± S.D. of three independent experiments.

Treatment	Lipid class							
	Sphingomyelins		Ceramides		Glucosylceramides		Phosphatidylcholines	
	mol %	% of control	mol %	% of control	mol %	% of control	mol %	% of control
NS siRNA (control)	7.7 ± 0.6	100 ± 7	0.36 ± 0.05	100 ± 15	0.27 ± 0.03	100 ± 12	36.4 ± 4.4	100 ± 12
LA siRNA	7.5 ± 0.2	97 ± 2	0.32 ± 0.04	87 ± 10	0.29 ± 0.07	108 ± 27	36.7 ± 1.1	101 ± 3
SMS1 siRNA	6.3 ± 0.2	81 ± 3	0.66 ± 0.10	182 ± 27	0.86 ± 0.20	318 ± 73	37.5 ± 4.0	103 ± 11
SMS2 siRNA	6.6 ± 0.3	86 ± 4	0.57 ± 0.11	165 ± 31	0.36 ± 0.03	136 ± 12	40.8 ± 1.8	112 ± 5
SMS1 + -2 siRNA	6.2 ± 0.3	82 ± 4	0.60 ± 0.04	167 ± 12	0.68 ± 0.03	250 ± 12	41.1 ± 4.3	116 ± 12

Both SMS1 and SMS2 Are Required for Growth in HeLa Cells— Previous work suggested that SM synthesis is essential for cell growth in mammalian cells (3, 4). Whether SMS1, SMS2, or both enzymes play a role in this process is unknown. To address this issue, we analyzed the effect of NS, LA, SMS1, and SMS2 siRNA treatment on the growth and viability of HeLa cells cultured in Opti-MEM containing 3% normal, 3% delipidated, or no fetal calf serum. Transfection with LA or NS siRNAs had no significant effect on cell growth or viability compared with mock-transfected cells.[4] In contrast, growth of cells treated with SMS1 siRNAs ceased within 3 days after transfection regardless of the culture conditions (Fig. 10, *A* and *B*). Propidium iodine staining revealed that depletion of SMS1 caused a slight reduction in cell viability compared with LA-depleted cells (from 98 ± 1 to 94 ± 2%). This drop in viability was accompanied by a 2-fold increase in the number of apoptotic (annexin V-positive/propidium iodine-negative) cells, namely from 3.3 ± 0.7 to 7.5 ± 1.5%. The percentage of apoptotic cells did not further increase upon prolonged treatment with SMS1 siRNA. Cells treated with SMS2 siRNA also displayed a growth defect, a reduction in viability (from 98 ± 1 to 92 ± 3%), and a 2-fold increase in cells undergoing apoptosis (from 3.3 ± 0.7 to 7.1 ± 1.1%). The growth arrest in SMS2-depleted cells occurred within 2 days of siRNA treatment, was independent of serum, and could be induced by any of three different SMS2 siRNAs that proved efficient in blocking cell surface-associated SM synthase activity (Fig. 10, *A* and *B*, and supplemental Fig. 2). From these results we conclude that HeLa cells require both SMS1 and SMS2 to sustain growth.

Yamaoka *et al.* (4) reported the isolation of a mouse lymphoid cell line with diminished SM synthase activity and a defect in cell growth when cultured in serum-free medium. Growth of these cells could be restored by external addition of SM. As shown in Fig. 11, supplementing the culture medium with exogenous SM at concentrations sufficient to relieve the growth arrest in SM synthase-deficient mouse lymphoid cells did not restore growth in SMS1- or SMS2-depleted HeLa cells. These results suggest that a drop in SM levels is not the sole or primary cause of the growth defect in SM-deficient HeLa cells.

DISCUSSION

We reported previously that the mammalian genome contains two conserved SMS genes, SMS1 and SMS2 (15). In this study we showed that SMS1 and SMS2 function as the principal Golgi- and plasma membrane-associated SM synthases in human cervical carcinoma HeLa cells, respectively. Moreover SMS1 and SMS2 each contributed to SM production *in vivo* and turned out to be essential for growth in HeLa cells. Although both SMS1- and SMS2-depleted cells displayed a reduced SM content, external addition of SM did not restore growth. These results indicate that the requirement of SM synthases for growth in HeLa cells goes beyond formation of SM.

SMS1 is localized exclusively to the Golgi complex, and three lines of evidence argue that this enzyme is primarily responsible for the Golgi-associated SM synthase activity in HeLa cells. First overexpression of SMS1 led to a substantial increase in an SM synthase activity that co-fractionated with the Golgi-resident enzyme GlcCer transferase and segregated from the plasma membrane on a Percoll density gradient. This was in contrast to the situation in SMS2-overexpressing cells, which showed a marked increase in plasma membrane-associated SM synthase activity. Second HeLa cells treated with siRNA to specifically down-regulate SMS1 expression were devoid of the SM synthase activity that normally co-migrates with GlcCer transferase on a Percoll gradient; the latter activity was essentially unaffected in SMS2-depleted cells. Finally SMS1 depletion abolished vital staining of the Golgi complex with C_6-NBD-ceramide; this staining was unperturbed in SMS2-depleted cells.

C_6-NBD-ceramide vitally stains the Golgi complex of a wide range of cultured cells (30, 31), but the mechanism by which this lipid accumulates in the Golgi is unclear. Our present findings indicate that molecular trapping of C_6-NBD-ceramide at the Golgi requires SMS1. This suggests that the Golgi labeling is due to formation of C_6-NBD-SM rather than to C_6-NBD-ceramide itself. Because of its short chain fatty acid and absence of a polar head group, C_6-NBD-ceramide readily diffuses across cellular membranes and through the cytosol. Upon arrival in the Golgi lumen, C_6-NBD-ceramide will be converted into C_6-NBD-SM. The latter is resistant to interorganellar partitioning and cannot be back-exchanged from cells with BSA because addition of the charged phosphorylcholine head group will trap it on the luminal leaflet of the Golgi membrane (34). This scenario is consistent with the observation that C_6-NBD-ceramide labeling of the Golgi complex in yeast is blocked instantly by aureobasidin A (32), a potent inhibitor of the enzyme responsible for inositol phosphorylceramide synthesis in the Golgi lumen (35). However, our data do not exclude an alternative scenario, namely that SMS1 traps C_6-NBD-ceramide at the Golgi by creating a unique lipid composition that slows down its monomeric diffusion to other cellular organelles. Hence whereas our findings demonstrated that
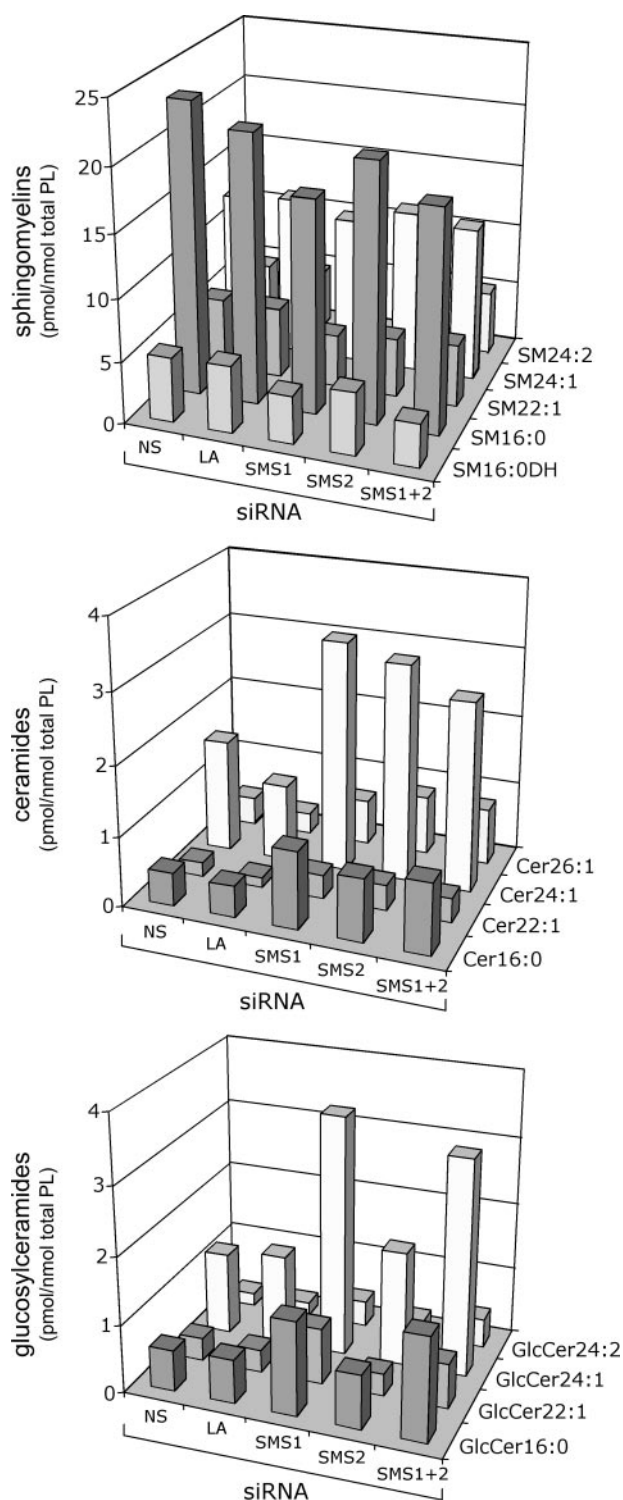
FIGURE 9. **Sphingolipid mass levels in control and SMS-depleted HeLa cells.** HeLa cells cultured in Opti-MEM containing 3% delipidated serum were treated for 7 days with NS, LA, SMS1, and/or SMS2 siRNA and then subjected to lipid extraction, liquid chromatography, and electrospray ionization tandem MS as described under "Experimental Procedures." Amounts of the most abundant molecular species of SM, ceramide (*Cer*), and GlcCer are given in pmol/nmol of total phospholipid (*PL*). Data shown are representative of three independent experiments. *DH*, *N*-palmitoyl-dihydrosphingosine moiety.

FIGURE 10. **Growth in HeLa cells is dependent on both SMS1 and SMS2.** *A*, HeLa cells plated at a density of $2 \times 10^5/9.6$-cm^2 dish were treated with LA, SMS1, and/or SMS2 siRNA in Opti-MEM without serum. At the indicated time points, viable cell numbers were assessed by the dye exclusion method. *B*, HeLa cells plated as in *A* were treated with LA, NS, SMS1, or SMS2 siRNA in Opti-MEM without serum or in Opti-MEM supplemented with 3% normal or delipidated serum. After 3 days, viable cell numbers were assessed by the dye exclusion method and expressed as the percentage of cell numbers in LA siRNA-treated cultures. Data shown are means ± S.D. of two independent experiments performed in duplicate. *delipid.*, delipidated.

FIGURE 11. **External addition of SM does not restore growth in SMS-depleted HeLa cells.** HeLa cells were treated with LA, NS, SMS1, or SMS2 siRNA in Opti-MEM supplemented with 3% delipidated serum in the absence (control) or presence of 80 μM SM or PC as described under "Experimental Procedures." After 3 or 7 days, viable cell numbers were assessed by the dye exclusion method and expressed as the percentage of cell numbers in LA siRNA-treated cultures. Data shown are means ± S.D. of three independent experiments performed in duplicate. *n.a.*, no addition.

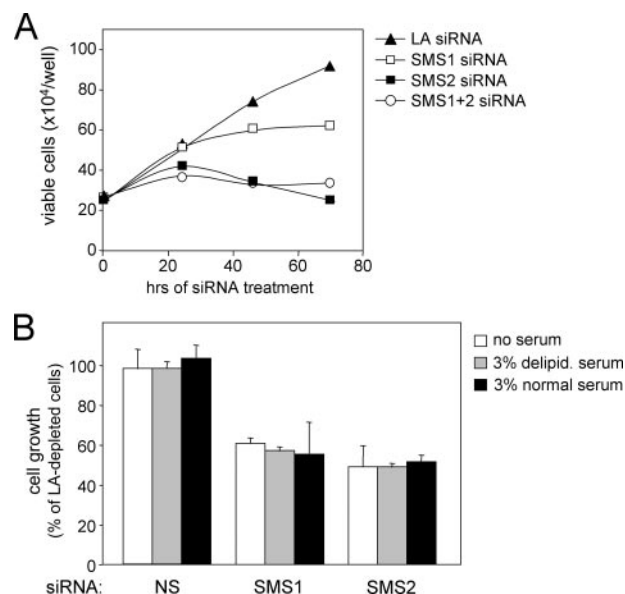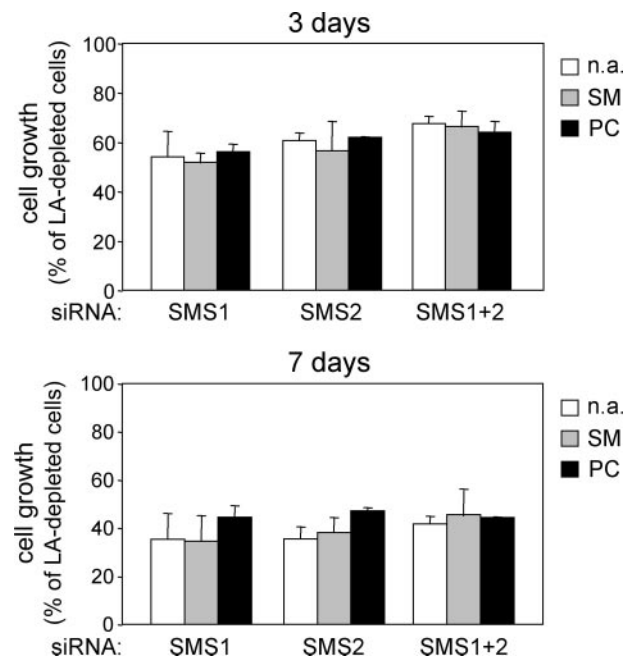C_6-NBD-ceramide staining of the Golgi requires SMS1, it remains to be established whether this staining involves production of C_6-NBD-SM or some preferential interaction between C_6-NBD-ceramide and endogenous lipids whose levels in the Golgi are (in)directly controlled by SMS1 (SM, DAG, and/or cholesterol). In any case, it appears that C_6-NBD-cera-

mide labeling of the Golgi provides a useful method to monitor SM synthase activity at this organelle *in situ*.

As reported previously for rat liver (33) and dog epithelial Madin-Darby canine kidney cells (29), we found that HeLa cells contain a plasma membrane-associated SM synthase activity. This activity was increased in SMS2-overexpressing cells, strongly reduced (~70% reduction) in SMS2-depleted cells, and essentially did not correlate with changes in SMS1 expression levels. Together with the finding that SMS2 primarily resides at the plasma membrane, these observations indicate that SMS2 operates as the major if not only cell surface-associated SM synthase in HeLa cells. Because SMS2 is expressed in essentially all major tissues (15, 17, 36) and in a variety of mammalian cell lines (this study), we anticipate that the presence of a cell surface-associated SM synthase activity is a general feature of mammalian cells.

In vitro enzyme assays on lysates of siRNA-treated cells showed that SMS1 and SMS2 together are responsible for the bulk of SM synthase activity in HeLa cells with SMS1 acting as a major (60–80% of total) and SMS2 acting as a minor (20–40% of total) SM synthase activity. Metabolic labeling of siRNA-treated cells with three different SM precursors revealed that both SMS1 and SMS2 contribute to SM production *in vivo* with a primary role for SMS1. Although SMS2 is predominantly associated with the plasma membrane, a minor fraction of the protein resides in the Golgi complex (Ref. 15 and supplemental Fig. 1). This raises the question of whether SMS2-dependent SM production occurs in the Golgi, at the plasma membrane, or both. Besides a reduced SM content, SMS1-depleted cells showed a nearly 2-fold increase in ceramides as well as a 3-fold increase in GlcCer levels. The latter finding indicates that blocking SM production in the Golgi causes a diversion of ceramide to GlcCer synthesis, which takes place in the same organelle. Compared with SMS1-depleted cells, cells deficient in SMS2 showed a similar increase in ceramide levels but no appreciable accumulation of GlcCer. This suggests that SMS2-dependent SM production primarily occurs at the plasma membrane.

Given that SMS1 and SMS2 together are responsible for the bulk of SM synthase activity in HeLa cells, it was rather unexpected that prolonged SMS depletion caused only a minor (maximum, 20%) reduction in SM levels. This discrepancy cannot be explained by uptake of SM from the medium because cells treated with SMS siRNAs under serum-free conditions showed a similar reduction in SM content. An alternative explanation could be that mammalian cells are equipped with an SMS-independent pathway of SM synthesis. For example, Muehlenberg *et al.* (37) proposed a pathway of SM synthesis in which ceramide is first converted to ethanolamine phosphorylceramide (EPC) via transfer of the head group from PE. EPC is then converted to SM by stepwise methylation in a reaction analogous to the *S*-adenosylmethionine-dependent conversion of PE to PC. Because HeLa cells contain an EPC synthase activity,[5] we tested whether SMS-depleted cells utilize EPC methylation to compensate for a drop in SMS-mediated SM produc-

tion. However, liquid chromatography-MS analysis of cells metabolically labeled with d_4-ethanolamine and d_9-choline revealed no evidence for an EPC methylation pathway that contributes significantly to SM homeostasis.[5] Instead we anticipate that the limited impact of SMS depletion on cellular SM composition is due to the growth arrest, which sets in within 3 days of siRNA treatment and which is accompanied by a general down-regulation of phospholipid synthesis and a substantial reduction in SM turnover rate (up to 4-fold decrease in SMS1/2-depleted cells as indicated by pulse-chase labeling with d_9-choline).[4]

Remarkably we found that HeLa cells require both SMS1 and SMS2 for growth. Concomitant with a substantial decrease in SM production levels, SMS1- and SMS2-depleted cells displayed a reduced SM content as well as an accumulation of ceramides. Considering the potentially vital contribution of SM to the barrier function of the plasma membrane and the antimitogenic properties of ceramide (9), either condition may compromise a normal growth rate. Yamaoka *et al.* (4) reported that a mouse lymphoid cell line with diminished SM synthase activity was defective in growth when cultured in the absence of serum or externally added SM. These data strongly suggest that SM plays a critical role in cell growth. However, we observed that the growth defect in SMS-depleted HeLa cells occurred regardless of whether the culture medium was supplemented with serum or exogenous SM. Although this does not preclude a vital function of SM in cell growth, our data indicate that the requirement of SMS1 and SMS2 in this process is not limited to maintaining a critical level of SM. Moreover our data raise the question of why cells need two SM synthases for growth.

One explanation may be that SMS1 and SMS2 utilize different molecular species of ceramide and/or PC to create distinct pools of SM and/or DAG that each serves an essential role in growth. However, characterization of SMS1 and SMS2 *in vitro* thus far revealed no fundamental differences in enzymatic properties. Both enzymes recognize synthetic short chain (C_6-NBD) as well as naturally occurring long chain (C_{18} to C_{26}) ceramides as suitable substrates for SM synthesis (15). In addition, MS analysis did not show any significant difference in molecular species composition between the ceramides that accumulate in SMS1- and SMS2-depleted HeLa cells (this study). It therefore appears that SMS1 and SMS2 posses a common substrate specificity at least toward ceramides.

Instead our data indicate that cell growth requires SM synthases to operate simultaneously in the Golgi and at the plasma membrane. SM synthesis generates DAG, and it has been reported that the level of DAG in the *trans* Golgi is critical for protein transport to the plasma membrane, a process essential for cell growth. In particular, three related protein kinases, protein kinases D1, D2, and D3, are directed to Golgi membranes via DAG-specific cysteine-rich domains, and these kinases are required for efficient formation of Golgi-derived secretory vesicles (12, 38). Moreover fumonisin B1, an inhibitor of ceramide synthesis that indirectly prevents production of DAG and SM from ceramide and PC, has been found to inhibit protein kinase D recruitment and to block protein transport from the *trans* Golgi network to the cell surface (12).

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[5] P. Ternes *et al.*, manuscript in preparation.

Due to its location, SMS2 may complement SMS1 by maintaining an appropriate level of SM at the plasma membrane but probably not with respect to liberating DAG for secretory vesicle formation at the Golgi. The requirement of SMS2 in cell growth may reflect a role in signal transduction at the plasma membrane. SMase-catalyzed hydrolysis of SM in response to external stimuli generates ceramide, a negative regulator of cell growth (14). By converting ceramide back to SM, SMS2 may attenuate SMase-induced signaling at the plasma membrane. This reaction would produce DAG, which is a well established mitogenic factor (39). Hence SMS2 may serve a critical role in cell growth by regulating the balance between pro- and anti-mitotic stimuli at the plasma membrane.

The widespread multiplicity of SM synthase genes in organisms generating SM suggests that it serves a fundamental role. Here we have demonstrated that this multiplicity is essential for growth in human HeLa cells. The mechanisms by which SMS1 and SMS2 contribute to cell growth will be the subject of future studies.

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