Sphingomyelin synthase-related protein SMSr controls ceramide homeostasis in the ER

Ana M. Vacaru,1 Fikadu G. Tafesse,1 Philipp Ternes,1 Vangelis Kondylis,3 Martin Hermansson,4 Jos F.H.M. Brouwers,2 Pentti Somerharju,4 Catherine Rabouille,3 and Joost C.M. Holthuis1

1Membrane Enzymology, Bijvoet Center and Institute of Biomembranes, and 2Biochemistry and Cell Biology, Faculty of Veterinary Medicine and Institute of Biomembranes, Utrecht University, 3584 CH Utrecht, Netherlands
3The Cell Microscopy Center, Department of Cell Biology and Institute of Biomembranes, University Medical Center Utrecht, 3584 CX Utrecht, Netherlands
4Medical Biochemistry, Institute of Biomedicine, University of Helsinki, Helsinki 00014, Finland

Ceramides are central intermediates of sphingolipid metabolism with critical functions in cell organization and survival. They are synthesized on the cytosolic surface of the endoplasmic reticulum (ER) and transported by ceramide transfer protein to the Golgi for conversion to sphingomyelin (SM) by SM synthase SMS1. In this study, we report the identification of an SMS1-related (SMSr) enzyme, which catalyses the synthesis of the SM analogue ceramide phosphoethanolamine (CPE) in the ER lumen. Strikingly, SMSr produces only trace amounts of CPE, i.e., 300-fold less than SMS1-derived SM. Nevertheless, blocking its catalytic activity causes a substantial rise in ER ceramide levels and a structural collapse of the early secretory pathway. We find that the latter phenotype is not caused by depletion of CPE but rather a consequence of ceramide accumulation in the ER. Our results establish SMSr as a key regulator of ceramide homeostasis that seems to operate as a sensor rather than a converter of ceramides in the ER.

Introduction

Sphingolipids are vital components of cellular membranes in organisms ranging from mammals to yeast. Besides providing a structural framework for plasma membrane (PM) organization, sphingolipids are dynamic regulators of a wide range of cellular processes. Sphingoid long-chain bases (LCBs), ceramides, and other intermediates of sphingolipid metabolism act as signaling molecules in the regulation of cell growth, death, migration, and membrane trafficking (Spiegel and Milstien, 2003; Hannun and Obeid, 2008). Notably, a sensitive balance between phosphorylated LCBs and ceramides, referred to as the LCBP/ceramide rheostat, appears critical for normal cell growth and lifespan (Mandala et al., 1998; Kobayashi and Nagiec, 2003). Moreover, sphingolipids form gradients along the secretory pathway that may affect protein sorting through hydrophobic matching of membrane spans (Bretscher and Munro, 1993; Holthuis et al., 2001; Patterson et al., 2008).

Given their impact on cell organization and survival, the local concentration of sphingolipids and their metabolic intermediates must be tightly controlled. Although the mechanisms that regulate membrane sterol concentrations are well established (Goldstein et al., 2006), little is known about the mechanisms controlling sphingolipid homeostasis. As ceramides constitute the backbone of all sphingolipids and directly participate in cellular life and death decisions (Morales et al., 2007), controlling their local concentration is critical. Ceramides can be generated by the breakdown of sphingomyelin (SM) through SMases at the PM (Andrieu-Abadie and Levade, 2002) or synthesized de novo by N-acylation of LCBs on the cytosolic surface of the ER (Mandon et al., 1992; Hirschberg et al., 1993). Nevertheless, blocking its catalytic activity causes a substantial rise in ER ceramide levels and a structural collapse of the early secretory pathway. We find that the latter phenotype is not caused by depletion of CPE but rather a consequence of ceramide accumulation in the ER. Our results establish SMSr as a key regulator of ceramide homeostasis that seems to operate as a sensor rather than a converter of ceramides in the ER.
reaction is controlled by components of TORC2 (target of rapamycin complex 2) presumably through phosphorylation of the ceramide synthase (Aronova et al., 2008).

In mammalian cells, the bulk of newly synthesized ceramides is converted to SM in the lumen of the Golgi (Tafesse et al., 2006). Efficient delivery of ER-derived ceramides to the site of SM production requires a cytosolic ceramide transfer protein (CERT; Hanada et al., 2003). Besides a START domain that binds ceramide, CERT contains an FFAT motif for interaction with the ER membrane and a pleckstrin homology domain targeting the Golgi. CERT is phosphorylated at a serine repeat motif, which down-regulates its ceramide transport function. Loss of SM and cholesterol from cells causes dephosphorylation of the motif, resulting in CERT activation (Kumagai et al., 2007). Thus, the local concentration of ceramides in cells is controlled by an intricate network of lipid-metabolizing enzymes, transfer proteins, kinases, and phosphatases.

SM production is mediated by a phosphatidylcholine (PC)/ceramide cholinephosphotransferase or SM synthase (SMS). Mammalian cells contain two SMS isoforms, SMS1 in the Golgi and SMS2 at the PM (Huitema et al., 2004). In addition, the mammalian genome encodes a third SMS-related (SMSr) protein of unknown function with homologues in nematodes and insects like Drosophila melanogaster (Fig. 1 A). Drosophila does not synthesize SM but produces the SM analogue ceramide phosphoethanolamine (CPE) as a major membrane constituent (Rao et al., 2007). CPE production also occurs in mammals and is catalyzed by a phosphatidylethanolamine (PE)/ceramide ethanolamine phosphotransferase or CPE synthase (CPES; Malgat et al., 1986, 1987). The identity of the responsible enzyme is not known. Production of SM and CPE involves a similar reaction chemistry (Malgat et al., 1986). Because SMS1, SMS2, and SMSr are structurally related and share two highly conserved sequence motifs with putative active site residues (Fig. 1 B; Huitema et al., 2004; Tafesse et al., 2006), SMSr is a prime candidate for the elusive CPES.

In this study, we show that SMSr displays CPES activity and, contrary to SMS1 and 2, localizes to the ER. However, we find that SMSr produces only trace amounts of CPE and that bulk production of CPE in insect cells is mediated by a different enzyme. Unexpectedly, blocking SMSr activity causes a marked increase of ceramide levels in the ER. This is accompanied by a fragmentation of ER exit sites and a structural collapse of the Golgi. These morphological aberrations are not caused by a lack of CPE but rather a consequence of the ceramide accumulation in the ER. We propose that SMSr is a CPES with dual activity as ceramide sensor to control ceramide homeostasis in the ER and that the latter process is critical for the integrity of the early secretory pathway.

**Results**

**SMSr proteins display CPES activity**

To test whether SMSr proteins catalyze CPE production, human SMSr (hSMSr) and Drosophila SMSr (dSMSr) were expressed in budding yeast, an organism lacking endogenous CPES activity. SMSr-expressing cells were lysed and incubated with fluorescent C2-NBD–ceramide (NBD-Cer). TLC analysis of the reaction mixtures showed the presence of an NBD-labeled product with a retention factor value distinct from that of NBD–ceramide phosphoinositol and NBD-SM. The product was missing in reactions performed with control (empty vector) cells (Fig. 1 C). Liquid chromatography (LC) mass spectrometry (MS) analysis identified the product as NBD-CPE (unpublished data), suggesting that SMSr proteins can synthesize CPE.

To determine whether SMSr proteins recognize natural ceramides as substrates for CPE production, a yeast strain was used in which the endogenous enzymes for ceramide production were replaced by mouse ceramide synthase CerS5 (Cerantola et al., 2007). As the ceramides produced in this strain structurally resemble those found in animal cells, they were expected to be suitable substrates for CPE biosynthesis. LC/MS/MS analyses revealed the presence of several molecular species of CPE in lipid extracts of both hSMSr- and dSMSr-expressing strains (Fig. 1 D). The extracts were devoid of SM. No CPE was detectable in control cells. Together, these results demonstrate that SMSr proteins function as CPESs.

**SMSr represents a major CPES activity in mammalian cells**

To investigate whether SMSr corresponds to the CPES activity previously described in mammalian cells (Malgat et al., 1986, 1987), lysates of HeLa cells in which hSMSr was overexpressed or depleted by RNAi were analyzed for CPES activity. Incubation of control cell lysates with NBD-Cer yielded three fluorescent products, corresponding to NBD-glucosylceramide (GlCer), NBD-SM, and NBD-CPE (Fig. 2 A, left lane). Although formation of NBD-CPE was stimulated by addition of PE, addition of either PC or CDP-ethanolamine had no effect, suggesting that PE is the headgroup donor in the CPES reaction (Fig. 2 B; Fig. S1; and see Fig. 4 E). Heterologous expression of V5-tagged hSMSr led to a sixfold increase in CPES activity, leaving SM activity unaffected (Fig. 2 A and C). Conversely, CPES activity dropped >50% after depletion of hSMSr by RNAi (Fig. 2 D). This shows that hSMSr represents a major CPES activity in HeLa cells.

To determine the contribution of SMSr to CPE biosynthesis in vivo, endogenous CPE production levels were monitored by metabolic labeling of various cell lines. Labeling human colon carcinoma Caco-2, liver carcinoma Hep-G2, or HeLa cells for 48 h with [14C]ethanolamine yielded only trace amounts of radiolabeled CPE (Fig. 2 F). The same was true when HeLa or CHO-K1 cells were labeled with [3H]phosphatidylethanolamine (Fig. 2 G). In contrast, labeling Drosophila S2 cells with [3H]ethanolamine showed that the CPE production level in these cells is much higher (Fig. 2 F, right). Indeed, MS analyses revealed that although S2 cells contain a substantial amount of CPE (15.3 ± 1.1 mol% of total phospholipid; n = 3), the CPE levels in HeLa or CHO-K1 cells are exceedingly low (~0.03 mol%, i.e., 300-fold lower than SM; see Fig. 5 A).

**SMSr produces only trace amounts of CPE**

Although mammalian cells contain a readily detectable CPES activity (Fig. 2 A), their CPE content is very low. One explanation for this discrepancy could be that SMSr resides in a compartment that is not easily reached by newly synthesized ceramide. To test this, the subcellular distribution of hSMSr-V5
Another explanation for the low CPE content could be the presence of an inhibitory factor or environment in the ER. We found that removal of the N-terminal sterile \( \alpha \) motif (SAM) domain of hSMSr caused its redistribution from the ER to the Golgi (Fig. 2 E and Fig. S2 B) but did not affect its enzymatic activity (see Fig. 4 A). However, metabolic labeling of HeLa cells expressing hSMSr showed no significant increase in CPE production levels compared with cells expressing the full-length protein (Fig. 2 F). Thus, the ER does not seem to impose an inhibitory environment on SMSr enzymatic activity.

Last, we investigated whether CPE is readily converted to another product. Although SM production in mammalian cells mainly occurs through SMS1-mediated headgroup transfer from PC to ceramide (Tafesse et al., 2006), an alternative pathway involves methylation of CPE (Muehlenberg et al., 1972). To investigate the relative contribution of the latter pathway...
over rate of CPE in HeLa cells was similar to that of SM (unpublished data). Thus, the low CPE content of mammalian cells is unlikely the result of CPE being a short-lived metabolic intermediate. Together, these results suggest that SMSr proteins are intrinsically unable to produce bulk amounts of CPE. This is consistent with our finding that CPE levels in SMSr-expressing yeast strains are invariably low (≤0.5 mol% of total phosphosphingolipid). As bulk amounts of CPE are present in Drosophila, to SM production, HeLa cells were metabolically labeled with D4-ethanolamine and D9-choline simultaneously. MS/MS analysis showed that the D4/D9 ratio of SM after 24-h labeling was ~1:100, indicating that headgroup transfer from PC is the major pathway of SM biosynthesis. Moreover, the D4-labeled SM pool most likely originated from headgroup transfer of D4-labeled PC, as this pool was reduced by depletion of human SMS1 (unpublished data). Finally, pulse-chase experiments with [14C]ethanolamine and [14C]choline showed that the turnover rate of CPE in HeLa cells was similar to that of SM (unpublished data). Thus, the low CPE content of mammalian cells is unlikely the result of CPE being a short-lived metabolic intermediate.

Together, these results suggest that SMSr proteins are intrinsically unable to produce bulk amounts of CPE. This is consistent with our finding that CPE levels in SMSr-expressing yeast strains are invariably low (≤0.5 mol% of total phosphosphingolipid). As bulk amounts of CPE are present in Drosophila,
we next addressed the contribution of dSMSr to CPE production in Drosophila S2 cells.

**Bulk production of CPE in insect cells is independent of SMSr**

We first analyzed the subcellular distribution of dSMSr in S2 cells by using an antibody that specifically cross reacted with dSMSr on immunoblots of S2 cell extracts (see Fig. 4 C). As the endogenous protein was hardly detectable by immunofluorescence microscopy, S2 cells were transfected with a Cu²⁺-inducible dSMSr construct, and expression of the protein was enhanced fivefold (as indicated by immunoblotting). dSMSr colocalized extensively with a GFP-tagged version of the ER marker PE N-methyltransferase (PEMT; PEMT-GFP) but not with the Golgi microtubule-associated protein dGMAP (Fig. 3, A and B). The ER residency of dSMSr was confirmed by quantitative immunoelectron microscopy (Fig. 3, C and D). As observed for hSMSr, removal of its SAM domain caused dSMSr to redistribute from the ER to the Golgi (Fig. S2, C–E), which is consistent with a fundamental role of SAM in ER retention of SMSr proteins.

We next investigated the contribution of dSMSr to CPE production in S2 cells. Incubation of S2 cell lysates with NBD-Cer led to formation of NBD-CPE (Fig. 4 A). NBD-CPE formation increased fivefold after overexpression of dSMSr. Mutation
did not enhance NBD-CPE production in HeLa cell lysates (Fig. 4 E, bottom).

In sum, insect cells contain two distinct CPESs: a PE/ceramide ethanolamine phosphotransferase corresponding to dSMSr and a dSMSr-unrelated CDP-ethanolamine/ceramide ethanolamine phosphotransferase that is likely responsible for bulk production of CPE. Importantly, these findings confirm that SMSr proteins in general lack the ability to produce bulk amounts of CPE.

Blocking SMSr catalytic activity causes ceramide accumulation in the ER

Although SMSr produces only trace amounts of CPE, SMSr-depleted HeLa cells showed a fourfold increase in ceramide levels (from 0.31 ± 0.01 to 1.29 ± 0.15 mol%; n = 3). This was accompanied by a threefold increase in GlcCer levels (from 0.32 ± 0.03 to 0.86 ± 0.09 mol%; n = 3; Fig. 5 A). Depletion of SMS1 caused a very similar rise in ceramide and GlcCer levels. In the latter case, however, this can be fully explained by a block in SM production (Tafesse et al., 2007). In contrast, the amount of ceramide and GlcCer accumulating in SMSr-depleted HeLa cells is likely due to the loss of CPE production through the SMSr-dependent pathway.
cells is 50-fold higher than can be ascribed to a block in CPE production (Fig. 5 A). The rise in ceramides affected all major species (Fig. S3 A and Table S1), could also be detected by [14C]serine labeling, and was not accompanied by an increase in sphingosine levels (Fig. S3 C). Moreover, it also occurred in SMSr-depleted hamster CHO-K1 and Drosophila S2 cells, although in the latter case to a lesser extent (Fig. S3 B).

As SMSr resides in the ER, the ceramide accumulation in SMSr-depleted cells likely originates from this organelle. This was confirmed by subcellular fractionation, which showed that the ER of SMSr-depleted HeLa cells contains nearly 300% more ceramide than the ER of lamin A (LA)–depleted control cells (Fig. 5, B and C). To verify this also for S2 cells, we created an ER-resident form of hSMS1 and analyzed its ability to reduce ceramide levels in dSMSr-depleted S2 cells. Consistent with a role of SMSr-derived SAM in ER retention, an hSMS1 swap mutant carrying the SAM-containing cytosolic tail of hSMSr, hSMS1Nr, localized primarily to the ER (Fig. 5, D and E). hSMS1Nr retained its SMS activity, as its expression in S2 cells supported production of SM (Fig. 5 F). Expression of ER resident hSMS1Nr but not Golgi-associated hSMS1 prevented the accumulation of [14C]serine-labeled ceramides in dSMSr-depleted S2 cells (Fig. 5 G). Together, these results indicate that SMSr acts as a negative regulator of ceramide levels in the ER.

To investigate whether SMSr requires its catalytic activity to prevent ceramide accumulation, dSMSr-depleted S2 cells were transfected with wild type or an enzyme-dead version of hSMSr. Expression of wild-type hSMSr effectively reduced the ceramide pool in dSMSr-depleted S2 cells (Fig. 5 H). However, mutation of active site residue Asp348 to Glu was sufficient to abrogate this effect. Transfection of hSMSr-depleted HeLa cells with siRNA-resistant hSMSr or hSMSrD348E yielded the same results (Fig. 5 H). Thus, even though SMSr produces only trace amounts of CPE, this appears essential to control the ceramide pool in the ER.

**Loss of SMSr perturbs the organization of the early secretory pathway**

Recent work revealed that CERT−/− mouse embryos accumulate ceramide in the ER and display a chronic state of ER stress characterized by vesiculation and engagement of the ER and up-regulation of downstream components of the unfolded protein response pathway (Wang et al., 2009). Even though SMSr-depleted S2 and HeLa cells show a substantial rise in ER ceramide levels, they did not display any of the morphological (ER dilation and vesiculation) or biochemical (XBP-1 splicing, induction of CHOP/GADD153, and up-regulation of ER chaperones) features of ER stress (unpublished data). Instead, we found that the organization of ER exit sites and the Golgi complex was greatly perturbed.

Golgi stacks in S2 cells do not form a large Golgi ribbon capping the nucleus as in mammalian cells. Instead, they remain discrete in the cytoplasm and are found in close proximity to transitional ER (tER) sites, forming 17–25 tER–Golgi units (Kondylis and Rabouille, 2003; Kondylis et al., 2007). In dSMSr-depleted S2 cells, the tER–Golgi units were much smaller and more numerous than in mock-depleted cells. Though the spatial relationship between the Golgi (marked by d120kd) and tER sites (marked by the COPII subunit dSec23) was maintained, both structures had a fragmented, hazy appearance (Fig. 6 A; and Fig. S4, A–I). This effect was quantitative, as more than half of the depleted cells displayed a fragmented Golgi (Fig. 7 C). However, the global architecture of the ER was unaffected by the depletion.

At the ultrastructural level, Golgi morphology in dSMSr-depleted S2 cells was profoundly affected. Instead of showing the typical organization of stacked cisternae (Fig. 6, B and D), the Golgi was converted into many short tubular structures in the majority of the cells (Fig. 6, C, E, and F). These tubular clusters were mostly devoid of KDEL-containing proteins but were positive for dSec23 (Fig. 6, E and F), suggesting that they represent a mixture of Golgi and tER membranes.

hSMSr-depleted HeLa cells showed a very similar phenotype. The Golgi ribbon was completely fragmented in more than half of the cells as indicated by immunostaining against markers of medial (MannII–RFP) and trans-Golgi compartments (TGN46; Fig. 6 G; Fig. 7 C; Fig. S4, J–L). These results further strengthen the functional similarity between hSMSr and dSMSr. Moreover, the requirement of SMSr for Golgi integrity appears specific because the Golgi ribbon was unperturbed in hSMS1-depleted HeLa cells (Fig. 6 G).

We next tested whether fragmentation of the early secretory pathway could result in or be the result of inhibition of anterograde transport. To this end, we monitored the transport efficiency of the transmembrane protein Delta from the ER to the PM in S2 cells (Kondylis and Rabouille, 2003). Brefeldin A treatment blocked PM delivery of Delta and caused its accumulation in the ER. In contrast, PM delivery of Delta was not significantly impaired in dSMSr-depleted S2 cells, suggesting that the fragmented tER–Golgi units in these cells are fully competent for anterograde transport (Fig. S5 A). Similar results were obtained in hSMSr-depleted HeLa cells using the well-established VSV-045G transport assay (Fig. S5, C and D). Moreover, SMSr depletion had no major impact on protein glycosylation (Fig. S5 E). Together, these findings indicate that SMSr proteins play a critical role in the acquisition of Golgi stack morphology without affecting the functional integrity of the organelle. This adds further evidence to the notion that transport of cargo to, through, and out of the Golgi complex does not require a fully developed organelle and can proceed under conditions in which the cisternal stacks are severely disorganized (Kondylis and Rabouille, 2003; Zolov and Lupashin, 2005).

**Golgi fragmentation in SMSr-depleted cells is caused by ceramide accumulation in the ER**

To test whether catalytic activity of SMSr is required to prevent Golgi fragmentation, we monitored the rescue of this phenotype by transfecting dSMSr-depleted S2 cells with wild-type or enzyme-dead hSMSr. Expression of wild-type hSMSr almost completely restored Golgi integrity. However, mutation of active site residue Asp348 to Glu was sufficient to abolish the rescuing effect (Fig. 7, A and C). These results completely parallel those obtained with hSMSr-depleted HeLa cells after transfection with siRNA-resistant hSMSr or hSMSrD348E (Fig. 7, B and C).

One possible interpretation of these results is that the structural integrity of the early secretory pathway depends on the trace...
Figure 5. **SMSr-depleted cells accumulate ceramides in the ER.** (A) HeLa cells treated with si LA, si hSMSr, or siRNA-targeting hSMS1 (si hSMS1) for 3 d were subjected to lipid extraction. Lipid classes were quantified by MS/MS analyses and expressed in mole percent of total lipid analyzed. Data shown are representative of three independent experiments. (B) HeLa cells expressing hSMS1-V5 were subjected to subcellular fractionation, yielding a PNS and fractions enriched for mitochondria (mito)/Golgi, PM/early endosomes (EE), or ER. Equal amounts of protein from each fraction were analyzed by immunoblotting. Note that the ER fraction was virtually free of other organelles except for a minor contamination with PM (<10%). (C) MS/MS analysis of ceramide and PE levels in ER fractions prepared from HeLa cells treated with si LA or si hSMSr for 3 d. (D) HeLa cells transfected with hSMS1-V5 or an hSMS1 swap mutant carrying the N-terminal tail of hSMSr, hSMS1Nr-V5, were double labeled for V5 and cis-Golgi marker GM130. (E) S2 cells cotransfected with hSMS1-V5 or hSMS1Nr-V5 and ER marker PEMT-GFP were labeled for V5. The white box marks a cell captured from a different field. Note that hSMS1Nr-V5 localizes to the ER and nuclear envelope (D and E, arrows). (F) S2 cells transfected with hSMS1-V5 or hSMS1Nr-V5 were labeled with [14C]choline for 5 h and subjected to lipid extraction, TLC, and autoradiography. (G) dSMSr-depleted S2 cells were transfected with hSMS1-V5 or hSMS1Nr-V5 and labeled for 5 h with [14C]serine. Levels of radiolabeled ceramides were determined by TLC and autoradiography. (H) dSMSr-depleted cells (S2/HeLa) were transfected with hSMSr-V5 or hSMSr D348E-V5 and labeled for 5 h with [14C]serine. Levels of radiolabeled ceramides were determined as in G. Transfection of HeLa cells was with siRNA-resistant (res) SMSr constructs. Untransf, untransfected. Error bars: (G) SD, n = 3; (C and H) range, n = 2.
Figure 6. **SMSr depletion disrupts tER–Golgi units in insect and mammalian cells.** (A) S2 cells were treated for 7 d with ds GFP, ds dSMSr #1, or ds dSMSr #2 and double labeled for dSec23 (tER sites) and d120kd (Golgi stacks). Confocal projections of the merge are presented. The single channels are presented in Fig. S5 A. Bar, 5 µm. (B–F) S2 cells treated for 5 d with ds GFP (B and D) or dSMSr #2 (C, E, and F) were fixed and processed for immunoelectron microscopy. Ultrathin cryosections were visualized before (B and C) or after double labeling for dSec23 (15-nm gold) and KDEL (10-nm gold; D–F). Note that Golgi stacks in most dSMSr-depleted cells are converted to a cluster of short tubular profiles (arrowheads) in cytoplasmic regions normally occupied by tER–Golgi units (between brackets). These short tubules are positively labeled for Sec23, suggesting that they comprise membrane of both compartments. G, Golgi stack. (G) Mannosidase II-RFP–expressing HeLa cells were treated with si LA, si hSMSr #2, or hSMS1 for 3 d and labeled for TGN46 and DNA (DAPI). Confocal projections of the merge are presented. The single channels are presented in Fig. S5 (J–L). Bars: (A and G) 5 µm; (B–F) 200 nm.
Figure 7. **Structural integrity of TIR–Golgi units requires an enzyme-active form of SMSr.** (A) S2 cells were treated with ds SMSr #2 and transfected with hSMSr-V5 or hSMSr\(^{D348E}-V5\). After 7 d, cells were double labeled for Golgi marker dGMAP and V5 to mark transfected cells. Confocal projections are presented. Note that cells expressing hSMSr (arrows) mostly show the typical wild-type Golgi organization of 17–25 large spots. In contrast, nontransfected cells or those expressing enzyme-dead hSMSr (arrowheads) typically have more numerous and smaller Golgi spots. (B) MannII-RFP–expressing HeLa cells were treated with si hSMSr #2 and transfected with siRNA-resistant (res) hSMSr-V5 or hSMSr\(^{D348E}-V5\). After 3 d, cells were labeled for V5 to mark transfected cells. Confocal projections are presented. Note that cells expressing hSMSr-V5 contain an intact Golgi (arrows). In contrast, cells expressing enzyme-dead hSMSr still have fragmented Golgi (arrowheads). (C) Quantitation of the rescue of the Golgi fragmentation phenotype in SMSr-depleted cells (HeLa/S2) after transfection of wild-type or enzyme-dead SMSr. SMSr-depleted cells are compared with control cells (si LA or ds GFP treated), and...
amounts of CPE produced by SMSr. Alternatively, as an enzyme-dead mutant of SMSr is unable to prevent ceramide accumulation in the ER (Fig. 5 H), fragmentation of the early secretory pathway in SMSr-depleted cells could be caused by the rise in ceramide levels in the ER. To distinguish between these possibilities, ER resident SMS hSMS1Nr was expressed in SMSr-depleted S2 or HeLa cells and analyzed for its ability to rescue Golgi fragmentation. Expression of hSMS1Nr partially restored Golgi integrity in both cell types (Fig. 7 D), indicating that acquisition of Golgi stack morphology is not strictly dependent on production of CPE. As expression of hSMS1Nr was effective in preventing ceramide accumulation in SMSr-depleted cells (Fig. 5 G), we asked whether blocking ceramide production or stimulating ceramide export from the ER would also suppress Golgi fragmentation. Treatment with ceramide synthase inhibitor fumonisin B1 or overexpression of CERT partially but reproducibly restored Golgi integrity in both SMSr-depleted HeLa and S2 cells (Fig. 7 D). This indicates that the structural collapse of the early secretory pathway in SMSr-depleted cells is the result of ceramide accumulation in the ER. Thus, these results further support a primary role of SMSr in controlling ceramide homeostasis in the ER.

Discussion

SMSr proteins define an unconventional class of sphingolipid synthases

In this study, we show that SMSr, the most conserved member of the multigenic SMS family, catalyzes production of the SM analogue CPE. SMSr likely accounts for the CPES activity described two decades ago in rat brain and liver microsomes (Malgat et al., 1986, 1987). Common features between these enzymes include the use of PE as headgroup donor, their ER residency, and a membrane topology in which the active site is facing the ER lumen. Although mammalian cells contain a readily detectable CPES activity, their CPE content is very low (i.e., 300-fold lower than SM). This enigma is neither caused by a limited supply of substrates to the enzyme’s active site nor by CMSr being a short-lived metabolic intermediate. Instead, we find that SMSr intrinsically lacks the ability to synthesize significant amounts of CPE. This is radically different from closely related SMS1, which catalyses bulk production of SM (Tafesse et al., 2007).

What makes SMSr so different from SMS1? SMS1 utilizes a lipid phosphate phosphatase–type reaction mechanism, which involves a single lipid-binding site and proceeds via transfer of phosphocholine from PC to a conserved histidine in the enzyme’s active site (Huijtema et al., 2004; Tafesse et al., 2006). When DAG is replaced by ceramide, the phosphocholine headgroup is transferred onto ceramide to form SM. The latter is then released from the active site to allow another round of catalysis. Mutational analysis of putative active site residues indicates that SMSr-mediated CPE production involves a very similar reaction mechanism. However, although SM readily dissociates from SMS1, we propose that CPE remains bound to the active site of SMSr, thus blocking a further round of catalysis. This could be the result of several factors. First, SMSr might have a higher affinity for CPE than for PE. Second, dissociation of SM from SMS1 might be facilitated by its preferential interaction with cholesterol. Contrary to SMS, CPE lacks this interaction (Terova et al., 2005). Thus, in the presence of cholesterol, SM production might be thermodynamically more favorable than CPE production.

The notion that SMSr is unable to produce significant amounts of CPE is further substantiated by our finding that bulk production of CPE in insects is mediated by a different enzyme. This second CPES uses CDP-ethanolamine instead of PE as headgroup donor, which is analogous to the ethanolamine phosphotransferases of the Kennedy pathway (Vance, 2008). This implies that, contrary to SM synthesis in mammals, bulk production of CPE occurs in the cytosolic leaflet of the membrane. As insects require CERT for efficient CPE production (Rao et al., 2007), the second enzyme likely resides in the Golgi.

SMSr qualifies for a ceramide sensor in the ER

Although SMSr is a poor CPES, we find that the enzyme has a major impact on ceramide levels in the ER. Indeed, SMSr-depleted mammalian cells display a rise in ceramide levels that is one order of magnitude too high to be explained by a reduced ceramide consumption for CPE biosynthesis. SMSr-depleted insect cells and SMSr-null mutants in Caenorhabditis elegans also show a marked increase in ceramide levels (this study; unpublished data). Strikingly, a single point mutation in the active site of SMSr proved sufficient to induce ceramide accumulation. This shows that SMSr must be catalytically active to keep ER ceramide levels low, even if the enzyme lacks the ability to consume significant amounts of ceramide. Together, these results establish SMSr as a novel regulator of ceramide homeostasis in the ER.

How does SMSr control ceramide levels in the ER? We anticipate that under normal conditions, only small amounts of ceramide will reach the enzyme’s active site. This is because ceramides synthesized on the cytosolic surface of the ER are continuously removed by CERT (Fig. 8 A). Consequently, only a minor fraction of SMSr enzymes will be trapped in a CPE-bound form. Yet when the rate of ceramide production exceeds ceramide removal by CERT, the pool of CPE-bound enzymes will expand. It is possible that CPE-bound SMSr adopts a conformation that relays a signal to inhibit ceramide biosynthesis and/or stimulate ceramide degradation (Fig. 8 B). Alternatively, SMSr-derived CPE might directly influence the activity of ceramide-metabolizing enzymes in the ER. Either feedback mechanism would ensure maintenance of ceramide homeostasis in the ER. Distinguishing between these possibilities will require reconstitution experiments with purified enzymes.
Collectively, our data suggest that SMSr is a CPES with a dual role as ceramide sensor to control ceramide homeostasis in the ER. As its active site faces the ER lumen, SMSr would only sense ceramides present in the exoplasmic leaflet, i.e., molecules that escaped CERT-mediated extraction at the cytosolic surface and flipped to the luminal side. Notably, this arrangement implies cooperativity between SMSr and CERT in keeping ER ceramide levels low. Thus, SMSr serves a biological role that is different from closely related SMS1 but analogous to the ensemble of membrane proteins that monitor sterol concentrations in the ER (Goldstein et al., 2006). In this respect, the SMS enzyme family displays an interesting parallel with the nutrient transporter family. Besides conventional transporters, the latter family also harbors transceptors that play a dual role as nutrient sensor because their conformational change during the transport process can trigger downstream signaling pathways (Holsbeeks et al., 2004).

**Functional implications of SMSr-controlled ceramide homeostasis**

Concurrent with the accumulation of ceramide in the ER, blocking SMSr activity causes a structural collapse of the early secretory pathway. The disruption of tER–Golgi organization completely parallels the rise in ER ceramide levels. Indeed, any condition that normalizes the ceramide concentration in the ER seems to restore Golgi stack morphology; i.e., the presence of enzymatically active SMSr, the consumption of ceramide by ER resident SMS, stimulation of ER export of ceramide by overexpression of CERT, or a block in ceramide production by ceramide synthase inhibitors. Conversely, blocking SM production to accumulate ceramides in the Golgi does not perturb its morphology.

How can an excess of ceramides in the ER affect Golgi organization? As the biogenesis of the Golgi is tightly linked to that of tER sites (Bevis et al., 2002), a disruption of tER sites likely contributes to the structural collapse of the Golgi. tER sites are thought to arise from patches of ER membrane proteins that have weak, cooperative affinity for one another (Bevis et al., 2002; Heinzer et al., 2008). These patches capture additional tER proteins until they grow large enough to produce COPII vesicles. A rise in ER ceramides may perturb the local physical environment required to stabilize patches of tER proteins. Alternatively, ceramides may affect tER site morphology by altering the membrane turnover of COPII components analogous to the situation in sterol-depleted cells (Runz et al., 2006). Moreover, ceramides serve as early mediators of apoptosis, an event accompanied by fragmentation of the Golgi complex. Apoptosis-induced Golgi fragmentation requires caspase 3–mediated cleavage of golgins, a family of coiled-coil proteins involved in maintenance of Golgi stack morphology (Lane et al., 2002). Interestingly, SMSr-depleted HeLa cells display hallmarks of apoptosis, including activation of caspase 3 (unpublished data). Whether blocking caspase activation prevents Golgi fragmentation in SMSr-deficient cells remains to be established.

A tight regulation of ceramide homeostasis in the ER likely has functional implications beyond sustaining the structural integrity of the early secretory pathway. Down-regulation of CERT sensitizes cancer cells to paclitaxel, suggesting that ER ceramides have functional implications beyond sustaining the structural integrity of the early secretory pathway. Down-regulation of CERT sensitizes cancer cells to paclitaxel, suggesting that ER ceramides are integral to taxane-induced cell death (Swanton et al., 2007). Moreover, CERT−/− mouse embryos accumulate ceramide in the ER and display a chronic state of ER stress (Wang et al., 2009). As components of the unfolded protein response are up-regulated during animal development (Reimold et al., 2000; Iwakoshi et al., 2007), a defective transport of ceramide might potentiate ER stress in the embryo (Wang et al., 2009). Whether loss of SMSr sensitizes cells to ER stress–inducing conditions is currently under investigation.

The ER is a highly efficient lipid distribution system that is intimately associated with other organelles, including mitochondria (Levine and Rabouille, 2005). Ceramides have been implicated in mitochondria-induced apoptosis and may reach these organelles via contact sites with the ER (Siskind et al., 2008; Stiban et al., 2008; Wang et al., 2009). The present identification of SMSr as a key regulator of ceramide homeostasis in the ER provides novel opportunities to explore the role of ceramides in mitochondria-induced cell death and for modulating taxane effects on tumors.
Materials and methods

Chemicals

NBD-Cer was obtained from Invitrogen, NBD-SM, POPE (2-oleoyl-1-palmitoyl-sn-glycerol-3-phosphoethanolamine), and POPC (2-oleoyl-1-palmitoyl-sn-glycerol-3-phosphocholine) were obtained from Avanti Polar Lipids, Inc. NBD-CPE was provided by P. Devaux (Institut de Biologie Physico-chimique, Paris, France). 2-14C[ethanol-1-2alpha]-hydroxycholesterol and 3-14C[serine were purchased from Amersham, Philadelphia, PA, and DMSO with [3H]phosphosine were obtained from MP Biomedicals. D2-ethanolamine and D2-choline were obtained from Cambridge Isotope Laboratories. All other lipids and chemicals were obtained from Sigma-Aldrich.

Antibodies

The following antibodies were used: rabbit polyclonal anti-V5 and mouse monoclonal anti-Flag (Sigma-Aldrich), mouse monoclonal anti-V5 (Invitrogen), mouse monoclonal anti-GM130 (BD), mouse monoclonal anti-KDEL, rabbit polyclonal anti-calnexin and anti-GRP78 (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-d120kd (EMD), rabbit polyclonal anti-dGOL-GIN243 (provided by S. Munro, Medical Research Council Laboratory of Molecular Biology, Cambridge, England, UK), rabbit polyclonal anti-Na+/K+-ATPase (C356-M09; provided by J. Koenderink, Radboud University Nijmegen, Nijmegen, Netherlands), rabbit polyclonal antiproliferation isomerase (provided by I. Braakman, Utrecht University, Utrecht, Netherlands), rabbit polyclonal anti-GFP (Invitrogen), mouse monoclonal anti-EEA1 (BD), rabbit polyclonal anti-DMAP (Kondylis et al., 2007), rabbit polyclonal anti-tissue transglutaminase (provided by PickCell Laboratories for the immunization of mice, yielding monoclonal anti-dSMSr antibody B5-G7.

DNA constructs

hSMSr, hSMS1, and dSMSr cDNAs were cloned into yeast expression vectors pYES2.1/V5-His-TOPO and mammalian expression vector pcDNA3.1/hSMSr, hSMS1, and dSMSr cDNAs were cloned into yeast expression vectors (Rao et al., 2007) were provided by M. Olayioye (University of Stuttgart, Stuttgart, Germany) and J. Acharya (National Cancer Institute at Frederick, Frederick, MD, respectively).

Yeast culture

Yeast strain 4Δlss5 (MATa ade2-101α strain his3-12 ltr2 200 leu2-3,112 lys2-801 trp1-142 ura3-52 lac1-1 TRP1 lac1-1::LEU2 ypc1::kanMX ypc1::kanMX4 trp1-200 leu2-3,112 lys2-801 amber ade2-101ochre his3-12 ltr2 200 ura3-52 lac1-1::LEU2 ypc1::kanMX ypc1::kanMX4 TRP1 ade2-101ochre his3-12 ltr2 200 ura3-52 lac1-1::LEU2 ypc1::kanMX ypc1::kanMX4 TRP1) was streaked onto YPD with or without 50 mg/ml G-418 (Invitrogen). HepG2 cells were grown in DME with 10% FBS (Cambrex) and CaCo-2 cells in DME-GlutamaxI with 10% FCS. Treatment with siRNA (QIAGEN) were performed as described previously in Tafesse et al. (2007).

Metabolic labeling

S2 cells [2.5 × 10^6] were labeled in 0.5 ml complete Schneider’s insect medium with 10 mM NBD-Cer, 1 µCi [3H]ethanolamine, 1 µCi [3H]choline, or 1 µCi [3H]serine at 27°C for the indicated time. Metabolically labeled membranes (10 cm dish; 50% confluency) were labeled in complete medium with 1 µCi [3H]ethanolamine or 1 µCi [3H]choline for the indicated time. Lipids were extracted in CHCl3/MeOH/10 mM acetic acid (1:4.4/0.2 [vol/vol/vol]) and processed according to Bligh and Dyer (1959). Half of the extract was subjected to mild alkaline hydrolysis. Radiolabeled lipids were analyzed by TLC in CHCl3/MeOH/25% NH4OH (50/25/25 [vol/vol/vol]). Lipids were detected by exposure to imaging screens (BAS-MAS).
Isolation of ER membranes
Hela cells were washed twice and harvested by scraping in ice-cold homogenization buffer (HB; 10 mM Heps, pH 7.8, 1 mM EGTA, 25 mM KCl, 1 mM protease inhibitor cocktail, and 1 mM PMSF) supplemented with 0.25 M sucrose. Cells were pelleted by centrifugation at 600 g and gently disrupted by a dounce homogenizer in 11 ml HB at 4°C. The homogenate was centrifuged twice at 500 average g (gmax, 5 min at 4°C) to generate a PNS. The PNS was centrifuged at 10,000 gmax (10 min at 4°C) to pellet mito-
chondria. The postmitochondrial supernatant was loaded onto a 1 ml 68% eMYC as described previously in Kondylis and Rabouille (2003). In brief, marked by an asterisk, whereas P > 0.001 is indicated (Fig. 7, C and D).

Each analyzing at least 400 (or ≥75 transfected) cells per condition. The phology. The averages were derived from three independent experiments, corresponding to numerous smaller labeled spots. Results were further processed using Photoshop software (version 7.0.1; Adobe).

Fig. S4 shows the single chan-
time points as indicated.

045G-GFP, kept at 39.5°C for 16 h, and chased at 32°C for different

treated with different siRNA constructs for 7 d, transfected with VSV-
fluorescence microscopy. For VSV-045G transport assay, HeLa cells were

absence of CuSO 4 for different time points. Subsequently, cells were
fixed, immunostained, and Delta arrival at PM was monitored by immuno-

isolation and purification of total lipides from animal tissues.

The fluorochromes used were DAPI, 

organelles except for a minor contamination with PM (<10%).

sphingosine. Ceramides are synthesized from sphingosine by the enzyme sphingosine kinase. The major species of ceramide but no sphingosine. Fig. S4 shows the single chan-
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