Sphingomyelin Synthase 1-generated Sphingomyelin Plays an Important Role in Transferrin Trafficking and Cell Proliferation

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Background: Sphingomyelin synthase (SMS) catalyzes the synthesis of sphingomyelin.

Results: Sphingomyelin-deficient cells failed to proliferate in response to transferrin. Transfection of SMS1 enabled these cells to generate sphingomyelin, promoting clathrin-dependent uptake of transferrin and its dependent proliferation.

Conclusion: SMS1 is indispensable for transferrin internalization and cell proliferation.

Significance: Our findings provide new insights into the role of SMS1 in transferrin biology.

Transferrin (Tf) endocytosis and recycling are essential for iron uptake and the regulation of cell proliferation. Tf and Tf receptor (TfR) complexes are internalized via clathrin-coated pits composed of a variety of proteins and lipids and pass through early recycling endosomes. We investigated the role of sphingomyelin (SM) synthases (SMS1 and SMS2) in clathrin-dependent trafficking of Tf and cell proliferation. We employed SM-deficient lymphoma cells that lacked SMSs and that failed to proliferate in response to Tf. Transfection of SMS1, but not SMS2, enabled these cells to incorporate SM into the plasma membrane, restoring Tf-mediated proliferation. SM-deficient cells showed a significant reduction in clathrin-dependent Tf uptake compared with the parental SM-producing cells. Both SMS1 gene transfection and exogenous short-chain SM treatment increased clathrin-dependent Tf uptake in SM-deficient cells, with the Tf being subsequently sorted to Rab11-positive recycling endosomes. We observed trafficking of the internalized Tf to late/endolysosomal compartments, and this was not dependent on the clathrin pathway in SM-deficient cells. Thus, SMS1-mediated SM synthesis directly Tf-TfR to undergo clathrin-dependent endocytosis and recycling, promoting the proliferation of lymphoma cells.

Transferrin (Tf) 4 is an important molecule regulating cell proliferation; it is the major iron-transport protein in serum and provides iron to cells by interacting with membrane Tf receptors (TfRs), such as TfR1 and TfR2 (1). Malignant cells highly express TfR and display a hyperactive Tf recycling system, reflecting their heightened need for iron for proliferation and DNA synthesis (2–4). Several studies have suggested that the TfR itself plays a role in cell proliferation (5–7). Thus, the Tf-TfR system appears to be crucial for cell proliferation.

The internalization of plasma membrane receptors and lipids occurs through the clathrin pathway and/or the lipid-raft endocytic pathway (8). Protein-lipid and protein-protein interactions are involved in the targeting of signaling molecules to specialized compartments in those pathways. Numerous studies have uncovered the proteins that participate in this machinery, including GTPases such as dynamins, coat components, and different adaptors (9–12). However, the role of lipid molecules remained relatively underappreciated by researchers until it was found that membrane lipids such as phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate are

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Regulation of Transferrin Trafficking by SMS1

important for this process (13–15). The importance of lipid molecules was further demonstrated by the finding that endocytosis is regulated by the catalytic action of phospholipid metabolizing enzymes, such as phospholipase D (16–18). Additional evidence for the vital role of lipids is the existence of lipid binding domains in numerous vesicle-forming proteins (19).

Sphingolipids such as sphingomyelin (SM) and ceramide have emerged as vital components of cellular membranes and are involved in diverse cell functions, including cell proliferation, death, and immunity (20). In contrast to phosphatidylinositol, their role in cellular trafficking has not been thoroughly investigated. We previously described the role of the SM cycle in cell differentiation and death, demonstrating a physiologically important role for the mutual conversion of SM and ceramide in cell proliferation (21, 22). SM is produced by the catalytic action of SM synthase (SMS), which is thought to be the sole enzyme responsible for SM synthesis in mammals (23). SMS catalyzes the transfer of phosphocholine from phosphatidylcholine to ceramide, resulting in the generation of SM and diacylglycerol. In mammals, two SMS isoforms (SMS1 and SMS2) have been shown to account for the synthesis of SM in the lumen of the trans-Golgi (24, 25). The human genome also contains an SMS-related gene (SMSr) whose product does not have the ability to synthesize SM but catalyzes the formation of the SM analog ceramide phosphoethanolamine (26, 27). Several lines of evidence indicate that SM formation by SMS is critical for cell proliferation and survival (25, 28, 29).

The SM recycling system utilizes the clathrin pathway like the Tf-TIR trafficking system (30). A small percentage of SM is trafficked to lysosomes where it is degraded to ceramide by acid sphingomyelinase (SMase) (31). Hydrolysis of plasma membrane SM by bacterial SMase (bSMase) rapidly induces the formation of numerous vesicles, probably containing ceramide, that pinch off from the plasma membrane in ATP-depleted macrophages and fibroblasts (32). Alterations in the sphingolipid composition of trafficking vesicles have the potential to affect the regulation of endocytosis and trafficking. In this study, we propose that SM for regulation of endocytosis and trafficking vesicles have the potential to affect the regulation of endocytosis and trafficking. In this study, we propose that SM for regulation of endocytosis and trafficking vesicles have the potential to affect the regulation of endocytosis and trafficking.
Regulation of Transferrin Trafficking by SMS1

**RESULTS**

Characterization of WR19L/Fas Cell Variants—To examine the role of SMSs in Tf trafficking and cell proliferation, we employed SM-defective murine lymphoma WR19L/Fas-SM(−) cells that were established from SM-rich WR19L/Fas-SM(+) cells (35). The parent cells, but not WR19L/Fas-SM(+) cells, constitutively expressed mRNA for SMS1 (Fig. 1A).

**Kinetics of Tf Uptake and Recycling**—For the Tf transport assay, cells were incubated in serum-free medium for 60 min at 37 °C to deplete intracellular Tf. The cells were then incubated with 1 μg/ml [125I]Tf at 37 °C for the indicated time periods. Free and plasma membrane-associated Tf were extracted by washing with PBS and acidic buffer (20 mM MES (pH 5.0), 137 mM NaCl, 50 μM desferal, and 0.1% BSA) at 4 °C. The amount of internalized [125I]Tf was determined using a gamma counter. For measurement of Tf uptake, cells were preincubated in serum-free medium for 60 min and treated with 20 μg/ml Alexa Fluor 488-conjugated Tf for 10 min. Cells were washed twice with ice-cold PBS and treated with acidic buffer for 10 min at 4 °C. Then cells were washed with ice-cold PBS and fixed with 1% paraformaldehyde for 20 min at 4 °C. Fluorescence was measured with a fluorescence-activated cell sorter (FACS).

**Immunofluorescence**—For the detection of plasma membrane SM, cells were washed twice and kept for 1 h in serum-free RPMI 1640 medium supplemented with 0.2% defatted BSA at 37 °C to eliminate any lipids acquired from the serum. Cells were cytospun onto slides and fixed with 1% paraformaldehyde. They were probed with lysenin-MBP and analyzed by confocal microscopy. Scale bars, 10 μm.

**Statistical Analysis**—Comparison between two groups was carried out using the unpaired Student’s t test.

![Image](https://example.com/image.png)
WR19L/Fas-SM(−) cells were stably transfected with human SMS1 or SMS2 genes to generate WR19L/Fas-SMS1 or WR19L/Fas-SMS2 cells, respectively. The respective mRNAs were detected in these cells (Fig. 1A). Next, we determined SMS activities in vitro and in vivo. WR19L/Fas-SM(−) cells were defective in in vitro (Fig. 1B) and in vivo (Fig. 1C) SMS activities, and cells overexpressing hSMS1 or hSMS2 showed in vitro SMS activities to a similar extent (Fig. 1B). The in vivo activity in WR19L/Fas-SMS1 cells was 150 pmol/10⁷ cells/h, similar to the parental WR19L/Fas-SM(−) cells. SMS2-overexpressing WR19L/Fas-SMS2 cells showed only a slight increase in activity compared with WR19L/Fas-SM(−) cells (Fig. 1C). Furthermore, we determined the cellular content of the SMS substrate, ceramide, and the product, SM, by LC/MS. Introduction of the SMS1 gene dramatically augmented SM content to 32.05 ± 3.23 pmol/nmol of lipid phosphate from 3.18 ± 1.85 pmol/nmol of lipid phosphate in WR19L/Fas-SM(−) cells, but SMS2-overexpressing WR19L/Fas-SMS2 cells displayed only modest increases in SM. The modest elevation in in vivo activity by SMS2 gene introduction and the robust increase in in vitro activity was closely correlated to SM levels in WR19L/Fas-SMS2 cells. However, there was no significant difference in cellular ceramide levels among these cells (Fig. 1D). Immunocytochemical detection of cell surface SM by lysenin showed that plasma membrane SM was detectable only in SMS1-expressing cells (WR19L/Fas-SMS1 cells; Fig. 1E). Thus, SMS1 appears to play an important role in forming SM, particularly at the plasma membrane.

**FIGURE 2.** SMS1-mediated SM synthesis promotes Tf-mediated cell proliferation. A, cells (1 × 10⁵ cells/ml) were kept in serum-free medium supplemented with 5 μg/ml Tf. After 24 or 48 h, viable cell numbers were counted by the trypan blue exclusion method. *, p < 0.001 versus WR19L/Fas-SM(−) cells. B, WR19L/Fas-SM(−) (black-filled column) or WR19L/Fas-SMS1 cells (red-filled column) were treated with 10 μM C₆-SM or 10 milliunits/ml bSMase, respectively. Cells were fixed, stained with MBP-conjugated lysenin, and analyzed by confocal microscopy. Scale bars, 10 μm. C, WR19L/Fas-SM(−) and WR19L/Fas-SMS1 cells were treated with or without 10 μM C₆-SM and then stimulated with Tf for 24 h. Viable cell numbers were counted by the trypan blue exclusion method. D, WR19L/Fas-SMS1 cells were treated with the indicated concentrations of bSMase and then incubated with Tf for 24 h. *, p < 0.001. Data were obtained from at least three independent experiments. Error bars, S.D.
Tf-induced cell proliferation requires SM at the cell surface and is dependent on the catalytic activity of SMS1. Tf and TfR complexes internalized via clathrin-coated pits pass through early endosomes and recycling endosomes, and this process is important for cell proliferation. To examine the involvement of clathrin-dependent internalization of Tf in cell proliferation, we employed a pharmacological approach using chlorpromazine (36, 37), which is known to suppress clathrin-dependent internalization. Chlorpromazine treatment inhibited Tf-induced cell proliferation of SMS1-expressing and SM-rich WR19L/Fas-SM(+/H11001) cells in a dose-dependent manner (Fig. 3). The TfR family consists of two isoforms, TfR1 and TfR2 (1, 38). As we failed to detect significant expression of TfR2 in WR19L/Fas-SM(+/H11001) cells (supplemental Fig. S1), TfR1 is likely the major isoform in these cells, accounting for Tf-induced cell proliferation. Thus, the data suggest that clathrin-dependent Tf-TfR1 internalization is important for cell proliferation.

Effects of Individual SMS Isoforms on Tf Internalization and Recycling—A recent study of trafficking organelles using synaptic vesicles as a model revealed that clathrin-containing vesicles are composed of various lipids, including SM (39). Thus, SM-deficient cells lacking SMSs were presumed to have impairments in clathrin-coated vesicle. Indeed, SMS-deficient cells were impaired in Tf-mediated cell proliferation (Fig. 2). We measured Tf uptake and recycling in all cell types. Cells were incubated with [125I]Tf for the indicated time periods. Tf uptake increased with time and then plateaued after 10 min, but amounts of Tf uptake after 10 and 20 min were significantly reduced in SM-deficient cells compared with SM-rich cells expressing SMS1 (Fig. 4A). Although cellular and/or cell surface expression of TfR1 possibly affected Tf uptake, there were no significant differences in expression levels between the WR19L/Fas variants (supplemental Fig. S1). These results suggest that SMS1-mediated SM synthesis is crucial for Tf uptake. We also determined the rate of Tf recycling in these cells. Cells were incubated with [125I]Tf for the indicated time periods. Tf uptake increased with time and then plateaued after 10 min, but amounts of Tf uptake after 10 and 20 min were significantly reduced in SM-deficient cells compared with SM-rich cells expressing SMS1 (Fig. 4A). Although cellular and/or cell surface expression of TfR1 possibly affected Tf uptake, there were no significant differences in expression levels between the WR19L/Fas variants (supplemental Fig. S1). These results suggest that SMS1-mediated SM synthesis is crucial for Tf uptake.
Regulation of Transferrin Trafficking by SMS1

Requirement of SMS1-mediated SM Synthesis for Clathrin-dependent Internalization of Tf—In light of the results above, SMS1-mediated SM synthesis likely controlled clathrin-dependent Tf internalization. To examine this possibility, we tested the effects of SM on clathrin-dependent endocytosis of Tf (Fig. 5A). K⁺ depletion (40) is known to abolish clathrin-dependent endocytosis, similar to chlorpromazine, thereby inhibiting Tf uptake. More than 50% of Tf internalization was sensitive to K⁺ depletion in both SM-rich WR19L/Fas-SM(+) and WR19L/Fas-SMS1 cells, whereas SM-deficient cells such as WR19L/Fas-SM(−) and WR19L/Fas-SMS2 showed less sensitivity to K⁺ depletion. Most importantly, the addition of 1 μM C6-SM significantly restored the susceptibility of SM-deficient WR19L/Fas-SM(−) cells to inhibition of Tf internalization by K⁺ depletion by more than 2-fold, suggesting that SM is a key lipid that allows Tf to be internalized through clathrin-coated vesicles.

Dynamin is a GTPase that is essential for clathrin-dependent vesicle formation (41), and the selective inhibitor dynasore has been shown to inhibit the clathrin-dependent internalization of Tf (42). Similar to K⁺ depletion, Tf internalization in SM-rich WR/Fas-SM(+) and SMS1 cells was sensitive to dynasore treatment compared with SM-deficient cells (Fig. 5B). Thus, SM appears to be required for dynamin-dependent internalization of Tf, and dynamin is unlikely to significantly contribute to the clathrin-independent pathway.

When internalized, Tf-TfR complexes are packed into clathrin-coated vesicles. To examine the effects of SMS1 introduction on the compartmentalization of Tf-TfR complexes into clathrin-coated vesicles, we performed confocal microscopy using Alexa Fluor 488-conjugated Tf and clathrin heavy chain antibodies. After 2 min of Tf chase, the colocalization of Tf with clathrin heavy chain in SM-deficient cells was 20.8%, and the restoration of SM by SMS1 introduction significantly increased colocalization (WR19L/Fas-SMS1 cells, 48.1%; Fig. 6, A and B).

No colocalization was observed after 5 min (data not shown), which matches the mode of Tf internalization and traffic in other cell types (41). Furthermore, we tested whether manipulating SM content at the plasma membrane affected the internalization of Tf with clathrin. The loading of exogenous C6-SM onto WR19L/Fas-SM(−) cells significantly restored early Tf/clathrin colocalization to 43.2% from 20.8%, and conversely, SM hydrolysis by bSmase led to the loss of early colocalization in WR19L/Fas-SMS1 cells, down to 20.8% from 48.1% (Fig. 6, A and B). No significant difference in protein levels of TfR1 and clathrin heavy chain between SM-rich and -deficient cells was found, suggesting that changes in protein expression were not involved in regulating the Tf internalization pathway from being primarily clathrin-dependent to being clathrin-independent or vice versa. Tf was colocalized with TfR1 in all WR19L/Fas variants (supplemental Fig. S1C), indicating that Tf compartmentalization is similar to that of TfR1. These data suggest that SMS1-mediated SM synthesis plays a key role in promoting the packaging of Tf-TfR1 complexes into clathrin-coated vesicles at the plasma membrane. Consistent with the results in Fig. 5, these results also suggest that SM deficiency directs Tf to the endocytic pathway instead of the clathrin pathway.

SM Synthesis by SMS1 Promotes Tf Sorting into Recycling Endosomes—Tf internalized along with other clathrin-coated cargo is sequentially sorted to Rab11-positive recycling endosomes (43). We examined the involvement of SMS1-mediated SM synthesis in the sequestration of Tf into these recycling endosomes. In SM-deficient WR19L/Fas-SM(−) cells, 9.4% of internalized Tf was colocalized with Rab11 at 5 min (Fig. 7), and no significant colocalization was observed at later time points (7 and 10 min, data not shown). Surprisingly, SM-rich WR19L/Fas-SMS1 cells showed significantly increased colocalization compared with SM-deficient WR19L/Fas-SM(−) cells. Those
results show that introduction of the SMS1 gene promoted the sorting of Tf into Rab11-positive compartments.

Treatment of SM-deficient WR19L/Fas-SM(−) cells with exogenous C₄-SM restored the colocalization of Tf with Rab11 by 28.7%, whereas bSMase hydrolysis of cell surface SM led to a decrease in Tf-Rab11 colocalization down to 26.0% from 36.4% in SM-rich WR19L/Fas-SMS1 cells, suggesting that SM promotes the sequestration of Tf to Rab11-positive recycling endosomes (Fig. 7). These results demonstrate that not only is SMS1-mediated SM synthesis required for promoting Tf pas-
sage thorough recycling endosomes but also that Tf exits from the recycling endosome pathway in SM-deficient cells.

**Tf Trafficking to the Lysosomal Degradation Pathway in SM Deficiency**—Cargo-specific sorting leads to distinct cargo trafficking. Cargo can be routed from early endosomes to late endosomes and lysosomes for degradation or to recycling endosomes that bring the cargo back to the plasma membrane. In SM-deficient cells, internalized Tf was less colocalized with Rab11-positive compartments compared with SM-rich cells, thereby passing into the degradation pathway. We examined possible routes of Tf movement to lysosomes in SM-deficient cells. Tf was colocalized with lysosomes (LysoTracker Red) in SM-deficient cells, and treatment of those cells with C6-SM decreased colocalization (Fig. 8A), whereas bSMase treatment of SM-rich cells increased colocalization (Fig. 8B), indicating that Tf might be degraded in the lysosomal compartment of SM-deficient cells. These results suggest that SM deficiency induces the sequestration of internalized Tf into the lysosomal compartment for degradation. Why the reduction by NH₄Cl treatment occurs in SM-rich cells remains unclear, but pH neutralization of the early endosomes by NH₄Cl is thought to inhibit the internalization of Tf.

**DISCUSSION**

We previously reported on the biological role of SMS, which is one of the most important enzymes regulating hematopoietic cell proliferation, differentiation, and death through “SM cycle” (21, 45–47). Up-regulation of SMS promotes cell proliferation in lymphocytes, hepatocytes, astrocytes, and fibroblasts (48–50). In contrast, inhibition of SMS activity is involved in inducing apoptosis of TNF-α-treated Kym-1 cells (51) and Fas-cross-linked Jurkat cells (47). Our group (25) and that of Holthuis and co-workers (24) discovered the SMS genes, but the biological role of SMS1 and SMS2 in Tf-mediated cell proliferation remained poorly understood. Here we demonstrate a role for SMS1-mediated SM synthesis in clathrin-dependent endocytosis of Tf-TfR1 complexes and cell proliferation. It also appears that the complexes on SM-deficient membranes could be redirected to lysosomal compartments for degradation.

Ding et al. (52) demonstrated that both SMS1 and SMS2 in THP1-derived macrophages are involved in generating plasma membrane SM. However, SMS2 introduction into WR19L/Fas-SM(−) cells showed little increase in in vivo SMS activity and SM levels, whereas enrichment of cellular and plasma membrane SM in SMS1-overexpressing WR19L/Fas-SMS1 cells resulted in increases in both in vivo and in vitro SMS activity (Fig. 1). Consistent with these results, mouse embryonic fibroblasts obtained from SMS2 knock-out mice, which still expressed SMS1, showed higher amounts of SM in the plasma.
Regulation of Transferrin Trafficking by SMS1

membrane compared with embryonic fibroblasts from SMS1 knock-out mice (data not shown), suggesting that SMS1 is the primary enzyme maintaining SM levels in the plasma membrane.

Why SMS2 overexpression in WR19L/Fas-SM(−) cells failed to fully generate SM in the plasma membrane remains unclear. It is possible that SMS2 activity is suppressed by an inhibitory molecule(s) in lymphoma cells, although the biological regulation of SMS2 activity remains poorly understood.

Tf and TIR are involved in the regulation of cell proliferation (53). Cells treated with anti-TIR antibodies could internalize Tf via the non-TfR/clathrin pathway, inhibiting cell proliferation (53). In addition, the inhibition of clathrin-dependent internalization of Tf suppressed cell proliferation (Fig. 3). Tf-TIR1 complexes are delivered to early endosomes where the low pH facilitates the dissociation of iron from receptor/ligand complexes (54), making iron biologically available. Iron-containing proteins (e.g. cytochromes, mitochondrial aconitate, and Fe-S proteins of the electron transport chain) catalyze key reactions involved in energy metabolism and DNA synthesis (e.g. ribonucleotide reductase) (54). In light of the requirement for iron in cell proliferation, the biological availability of this metal plays an important role in Tf-induced cell proliferation. Therefore, the present study suggests that SMS1-mediated SM synthesis plays a key role in generating the biological activity of Tf.

SM deficiency is likely to decrease Tf colocalization with Rab11 (Fig. 7) and redirects Tf to the lysosomal compartment (Fig. 8) without affecting the rate of Tf recycling (Fig. 4). Although these results seem to be inconsistent, we can speculate why SM deficiency had no effect on the Tf recycling rate. Internalized Tf may simply not pass through early endosomes. It has been shown that (a) internalized Tf is recycled directly back from early endosomes to the plasma membrane (55), and (b) Tf is recycled in the absence of perinuclear recycling endosomes (44). In WR lymphoid cells, the majority of internalized Tf might be transported from early endosomes to the plasma membrane. Thus, SM deficiency is suggested to have no effects on the rate of Tf recycling.

We propose that SMS1 activity accounts for the majority of SM synthesis. Although SM is a possible constituent of transport vesicles and plays a role in the clathrin-dependent trafficking of Tf, its mechanism of function still remains largely unknown. SMase hydrolyzes SM, generating ceramide. The levels of ceramide in SM-deficient cells were similar to those in SM-rich cells (Fig. 1). Furthermore, in contrast to SM in the present study, ceramide is known to prevent classical PKC-dependent sequestration of Tf into the recycling endosome-like compartment, the pericentriol, by activating ceramide-activated protein phosphatases (56). These rules out the involvement of ceramide. Thus, SM, but not ceramide, is likely to be the key lipid regulating the clathrin-dependent internalization of Tf.

SM deficiency disrupts the clathrin-dependent uptake of Tf. LDL and cholera toxin subunit B are internalized through clathrin-dependent and independent pathways, respectively (57). SM deficiency had no significant effects on their uptake (supplemental Fig. S3). Thus, SM is specifically required for the clathrin-dependent internalization of Tf in mouse lymphoma cells.

Plasma membrane receptors are not always degraded by lysosomes; some are recycled back to the plasma membrane after endocytosis (58). For example, Tf and TIR are internalized and recycled back to the cell surface through recycling endosomes. However, this does not imply that Tf/TIR complexes are always recycled and used endlessly. TIR itself undergoes degradation and is degraded by a Rab12-dependent pathway (59). Tf is directed to lysosomal compartments close to perinuclear regions in SM-deficient cells (Fig. 8A). Although we failed to detect the degradation products of Tf and/or TfR1 (data not shown), Tf-TIR1 complexes are presumed to undergo degradation. The molecular mechanisms underlying the redirection of Tf to lysosomal compartments in SM-deficient cells remain unknown.

In summary, SM synthesized by SMS1 plays a pivotal role in the clathrin-dependent internalization of Tf-TIR and promotes cell proliferation. SM deficiency (e.g. due to a lack of SMS1) facilitates the sequestration of Tf into endolysosomal compartments, thereby reducing the biological availability of Tf-derived iron and its ability to enhance cell division.

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Regulation of Transferrin Trafficking by SMS1