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Mammalian ORMDL Proteins Mediate the Feedback Response in Ceramide Biosynthesis*

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Deanna L. Siow[‡] **and Binks W. Wattenberg**[‡§1]

From the [‡]*James Graham Brown Cancer Center, Louisville, Kentucky 40202 and the* [§]*Departments of Medicine, Biochemistry and Molecular Biology, and Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, Kentucky 40202*

Background: The yeast Orm1/2 proteins regulate ceramide biosynthesis.
Results: Depletion of the mammalian Orm1/2 homologues, ORMDL1–3, eliminates the negative feedback of exogenous ceramide on ceramide biosynthesis in HeLa cells.
Conclusion: ORMDL proteins are the primary regulators of ceramide biosynthesis in mammalian cells.
Significance: Therapeutically manipulating levels of the pro-death lipid, ceramide, requires a molecular understanding of its regulation.

The mammalian ORMDL proteins are orthologues of the yeast Orm proteins (Orm1/2), which are regulators of ceramide biosynthesis. In mammalian cells, ceramide is a proapoptotic signaling sphingolipid, but it is also an obligate precursor to essential higher order sphingolipids. Therefore levels of ceramide are expected to be tightly controlled. We tested the three ORMDL isoforms for their role in homeostatically regulating ceramide biosynthesis in mammalian cells. Treatment of cells with a short chain (C6) ceramide or sphingosine resulted in a dramatic inhibition of ceramide biosynthesis. This inhibition was almost completely eliminated by ORMDL knockdown. This establishes that the ORMDL proteins mediate the feedback regulation of ceramide biosynthesis in mammalian cells. The ORMDL proteins are functionally redundant. Knockdown of all three isoforms simultaneously was required to alleviate the sphingolipid-mediated inhibition of ceramide biosynthesis. The lipid sensed by the ORMDL-mediated feedback mechanism is medium or long chain ceramide or a higher order sphingolipid. Treatment of permeabilized cells with C6-ceramide resulted in ORMDL-mediated inhibition of the rate-limiting enzyme in sphingolipid biosynthesis, serine palmitoyltransferase. This indicates that C6-ceramide inhibition requires only membrane-bound elements and does not involve diffusible proteins or small molecules. We also tested the atypical sphingomyelin synthase isoform, SMSr, for its role in the regulation of ceramide biosynthesis. This unusual enzyme has been reported to regulate ceramide levels in the endoplasmic reticulum. We were unable to detect a role for SMSr in regulating ceramide biosynthesis. We suggest that the role of SMSr may be in the regulation of downstream metabolism of ceramide.

Ceramide is both a proapoptotic signaling lipid and an essential metabolic intermediate in the formation of sphingomyelin and glycosphingolipids (reviewed in Ref. 1). The essential, but lethal, nature of ceramide requires tight control of cellular ceramide levels. The metabolism of ceramide is complex. Ceramide levels are elevated by *de novo* biosynthesis and degradation of sphingomyelin and glycosphingolipids. Ceramide levels are diminished by degradation to the sphingosine backbone by ceramidases and consumption by conversion to sphingomyelin and glycosphingolipids. The homeostatic mechanisms that maintain control of ceramide levels are only beginning to be understood. Upstream metabolic pathways are clearly important, but how, or even if, they are regulated homeostatically is unclear. However, progress has been made in elucidating the molecular control of *de novo* ceramide biosynthesis. Serine palmitoyltransferase (SPT)[2] is the committed and presumptive rate-limiting step in ceramide biosynthesis (2). Mammalian SPT consists of two core subunits (SPTLC 1 and 2) (reviewed in Ref. 3). Additionally, regulatory subunits have also been reported (4, 5). In yeast, Orm proteins (Orm1 and -2) associate with SPT and negatively regulate SPT activity in response to changes in cellular ceramide levels (6–8). The yeast Orm proteins are regulated by phosphorylation. Regulation of phosphorylation of the Orms is attributed to the ceramide-sensitive activity of the Ypk-1 kinase (9, 10) and to the ceramide-responsive phosphatase protein phosphatase 2A (10). Mammalian cells contain a homologous set of proteins, the ORMDLs (isoforms 1–3). One isoform, ORMDL3, has been strongly linked in genome-wide associate studies with elevated risk for asthma (11). However, because the function of ORMDL3 is unknown, the mechanism by which elevated ORMDL3 levels would impact on asthma has not been approached. Genetic knockdown of the ORMDL proteins results in elevated ceramide levels (6). However, the mechanism by which the ORMDL proteins regulate ceramide levels and whether they are responsive to changes in cellular ceramide content have not been established. Mammalian cells also express a member of the sphingomyelin synthase family, SMSr, which has been implicated as a ceramide sensor in ceramide homeostasis (12). SMSr generates phosphoethanolamine-ceramide rather than sphingomyelin, is a low efficiency enzyme and, unlike the conventional sphingomyelin synthases, is localized in the endoplasmic reticulum rather than in the Golgi apparatus. Depletion of SMSr results in

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[1] To whom correspondence should be addressed: University of Louisville School of Medicine, Clinical and Translational Research Bldg., 505 South Hancock St., Louisville, KY 40202. Tel.: 502-852-7762; Fax: 502-852-3661; E-mail: b0watt01@louisville.edu.

[2] The abbreviations used are: SPT, serine palmitoyltransferase; ER, endoplasmic reticulum; HMEC, human mammary epithelial cells; FB1, Fumonisin B1.

elevated ceramide levels in the endoplasmic reticulum (ER). The data of Vacaru *et al.* (12) suggest an intriguing model in which the SMSr production of phosphoethanolamine ceramide acts as a ceramide sensor in the ER. The downstream effectors of phosphoethanolamine ceramide have not been identified. Here, we establish that the mammalian ORMDL proteins control ceramide biosynthesis under normal growth conditions and are completely responsible for the negative feedback response of ceramide *de novo* biosynthesis to exogenous ceramide. Further, we find that all three ORMDL isoforms are necessary for this response. Additionally, we demonstrate that the response of SPT activity to exogenous ceramide can be recapitulated in permeabilized cells and use that system to demonstrate that ORMDL proteins directly regulate SPT activity. Finally, we were unable to detect a role for SMSr in the regulation of ceramide biosynthesis.

EXPERIMENTAL PROCEDURES

Materials—Silencer® Select siRNA oligonucleotides for human ORMDL1 (s41257), ORMDL2 (s26474), ORMDL3 (s41262), and SMSr (SAMD8 s44495) were from Ambion®. Lipofectamine® RNAiMAX transfection reagent and TRIzol® were from Invitrogen. Ambion®, Applied Biosystems®, and Invitrogen are all part of Life Technologies. L-[^{3}H(3)]-Serine, scintillation vials, and scintillation fluid were from PerkinElmer Life Sciences. D-[*erythro*-3-^{3}H]Sphingosine was from American Radiolabeled Chemicals (St. Louis, MO). Whatman thin layer chromatography (TLC) plates were from VWR International (Radnor, PA). C6-ceramide, C16-ceramide, and D-*erythro*-sphingosine were from Avanti Polar Lipids (Alabaster, AL). Fumonisin B1 was from Cayman Biochemicals (Ann Arbor, MI). All tissue culture cell lines used were from ATCC (Manassas, VA). Cell culture media and supplies were from VWR International and Lonza (Atlanta, GA). Organic solvents were from Thermo Fisher Scientific. All other chemicals used were from Sigma-Aldrich unless otherwise indicated.

Cell Culture and Transfections—HeLa (human cervical carcinoma) and A549 (human lung carcinoma) cells were cultured in high glucose DMEM containing 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. PC-3 (human prostate adenocarcinoma) cells were cultured in Ham's F-12 minimum Eagle's medium containing 10% FBS, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Human mammary epithelial cells (HMEC) were cultivated in MEBM^{TM} supplemented with MEGM^{TM} SingleQuot kits (Lonza). All cell lines were maintained at 37 °C and 5% CO_2 and passaged when ~80–90% confluent. For gene-specific, siRNA-mediated knockdowns, $6-8 \times 10^4$ cells/well were plated in collagen-coated 12-well plates using 2 ml of complete medium overnight. Transient transfections on all cell lines were carried out using Lipofectamine® RNAiMAX transfection reagent per the manufacturer's recommendations and siRNA oligonucleotide concentrations ranging from 5 to 15 nM as indicated in each figure legend. Transfections were carried out for 24–72 h depending on the subsequent treatment protocol. Unless otherwise specified, cells were grown in serum-containing medium prior to measurement of sphingolipid biosynthesis. siRNA transfections resulted in between 72–94% depletion of ORMDL mRNA and 75–82% depletion of SMSr mRNA as measured by real-time PCR (data not shown).

C6-ceramide Treatment and Radiolabeling Cells with ^{3}H Substrates—Following siRNA-mediated knockdown, medium was removed, and cells were washed with PBS. Cells were treated with C6-ceramide, 10 μM final concentration (from a 1 mM solution complexed with 2% fatty acid-free BSA), in 1 ml/well antibiotic-free culture medium for 2 h at 37 °C and 5% CO_2. After treatment was complete, media were removed, and cells were washed with PBS. Serine-free media containing 1% dialyzed FBS and 10 μCi/ml [^{3}H]serine or 0.15 μCi/ml [^{3}H]sphingosine were used for radiolabeling cells at 1 ml/well volume. Cells were labeled for either 60 min ([^{3}H]serine) or 30 min ([^{3}H]sphingosine). At the end of each time point, labeling media were removed, and cells were washed once with PBS. For lipids, cells were harvested directly on the plates with acidic methanol as described below.

Lipid Extraction and Measurement of ^{3}H Incorporated into Ceramides—At the completion of the labeling protocol, cells were harvested directly on the plates with 2×900 μl of acidic methanol (150:1, v/v) and scraping and then transferred to 13 × 100-mm glass tubes and sonicated in a bath sonicator to break up cell clumps. Total cellular lipids were extracted using a modified version of the method of Bligh and Dyer (27) as reported previously (13). The bottom organic phase was collected and dried under nitrogen and then spotted onto thin layer chromatography plates Partisil K6 (Silica Gel 60 Å, Whatman). Radiolabeled ceramides were resolved using chloroform:acetic acid:MeOH (90:10:2, v/v) as the development system. C16-ceramide was identified using a known standard applied with each sample and iodine staining. Bands corresponding to C16-ceramide were scraped into 7-ml scintillation vials and vortexed with 3 ml of scintillation fluid prior to reading. Incorporation of ^{3}H into C16-ceramide was quantitated using a Tri-Carb 2010TR scintillation counter (PerkinElmer Life Sciences). Results are model reported as mean cpm/mg of total protein or mean cpm/well.

SPT Assay in Permeabilized Cells—HeLa cells were plated onto collagen-coated 24-well plates at 7×10^4 cells/well in 2 ml of medium. The next day, cells were transfected with siRNA oligonucleotides at 15 nM total siRNA/well with 0.6 μl/well Lipofectamine® RNAiMAX (Invitrogen). For ORMDL knockdown, siRNA oligonucleotides (listed above) were used at 5 nM each per well. 24 h later, cells were washed with PBS, and 200 μl/well of Opti-MEM (Gibco) containing 200 μg/ml digitonin (Sigma) was added; cells were incubated for 3 min at 37 °C. The permeabilization medium was removed, and cells were washed with PBS. 200 μl of preincubation medium (50 mM HEPES, pH 8.0, 1 mM ATP, 1 mM $MgCl_2$, 20 μM pyridoxyl-5′-phosphate, 200 μM NADPH, 5 mM glucose 6-phosphate, 5 IU/ml glucose-6-phosphate dehydrogenase) was added with or without 20 μM C6-ceramide (added from a 1 mM stock complexed to 2% fatty-acid free BSA in PBS) and/or 1 μM myriocin. Cells were incubated for 20 min, and then an additional 200 μl/well of incubation medium was added without removing the preincubation medium. Incubation medium was identical to the preincubation medium with the addition of (final concentration) 4 μCi of [^{3}H]serine/well, 0.5 mM serine, and 25 μM palmitoyl-CoA. Cells were incubated for an additional 60 min. Cells were then

extracted without removing the incubation medium by a modification of the method of Rutti *et al.* (14) by adding 0.4 ml of alkaline methanol (0.7 KOH/100 ml of MeOH) and transferring cells into a 2-ml screw-cap microcentrifuge tube. To finish lipid extraction, 0.1 ml of $CHCl_3$ was added, and the extract was vortexed; 0.5 ml of additional $CHCl_3$ was added along with 0.3 ml of alkaline H_20 (0.1 ml of 2 N NH_4OH/100 ml of H_20) and 0.1 ml of 2 N NH_4OH followed by vortexing. Samples were centrifuged for 1 min at 16,000 × *g* in a microcentrifuge. The top phase was removed by aspiration, and 1 ml of alkaline H_20 was added and vortexed followed by centrifugation as above. The top phase was removed, and the alkaline H_20 wash was repeated. Finally, 350 μl of the bottom, organic phase was removed to 7-ml scintillation vials, and organic solvent was removed by a gentle stream of N_2. 3 ml of scintillation mixture was added, and counts were determined by liquid scintillation counting as described above.

Labeling and Extraction for Total Sphingolipids—HeLa cells were plated and treated with control and anti-ORMDL siRNA in 12-well plates as described above for 48 h. Where indicated, cells were incubated overnight with 100 μM fumonisin B1 in complete medium before treatment. Cells were then treated with either 10 μM C6-ceramide or 10 μM sphingosine complexed to 2% fatty acid-free BSA for 2 h before labeling with 0.5 ml of [^{3}H]serine-containing medium for 1 h. Serine-free medium (as outlined above) contained 10 μCi/ml [^{3}H]serine and, where indicated, 1 μM myriocin. At the conclusion of labeling, cells were washed with ice-cold PBS and harvested into 0.75 ml of alkaline MeOH (0.7 g of KOH/100 ml of MeOH). An additional 0.75 ml of alkaline MeOH was added to each well and pooled with the scraped cells. The cell suspensions were then sonicated in a bath sonicator to disperse the cells. Extracts were then incubated for 30 min at 37 °C to hydrolyze glycerolipids. 0.6 ml of alkaline H_20 (100 μl of 2 N NH_4OH/100 ml of H_20) and 0.7 ml of $CHCl_3$ were then added, and the suspension was vortexed. The phases were then broken by adding 0.675 ml of alkaline H_20, 225 μl of 2 N NH_4OH, and 0.75 ml of $CHCl_3$. The extract was then vortexed for 1 min and centrifuged to clear the phases. The bottom, organic phase was removed and washed two times with 1.5 ml of alkaline H_20. 500 ml of the washed organic phase was dried in scintillation vials, and radioactivity was determined as described above.

RESULTS AND DISCUSSION

It has long been known that there are feedback mechanisms that homeostatically control *de novo* biosynthesis of ceramide in mammalian cells (15). Here, we test whether the ORMDL proteins, mammalian homologues of the yeast Orm proteins, have a role in this regulation. We also test the role of the atypical sphingomyelin synthase protein, SMSr, in this homeostatic regulation.

ORMDL Proteins Mediate the Response of HeLa Cells to Exogenous C6-ceramide—To directly measure the rate of ceramide biosynthesis, we employed labeling of sphingolipids with [^{3}H]serine under conditions where incorporation of the label into ceramide is linear with respect to time (not shown.) Monolayers of HeLa, A549, and HMEC cells were transfected with siRNAs targeting all three ORMDL isoforms simultaneously or a scrambled control siRNA. After 48 h, cells were treated with 10 μM C6-ceramide for 2 h and then labeled with [^{3}H]serine for 1 h. Lipids were extracted, and ceramide was isolated by thin layer chromatography and quantitated as described under "Experimental Procedures." As seen in the untreated samples (Fig. 1, *A–C*, controls), knockdown of all three ORMDL proteins resulted in increased levels of [^{3}H]serine incorporated into ceramides. This result directly demonstrates in multiple cell types that even under normal growth conditions, the ORMDL proteins are negative regulators of ceramide biosynthesis. This result complements the study of Breslow *et al.* (6), which demonstrated that depletion of the ORMDL proteins results in an increase in steady-state ceramide levels, but did not directly measure biosynthesis. As is well established, (15) the addition of C6-ceramide to cells strongly represses ceramide biosynthesis (Fig. 1, *A–C*). This inhibition is almost completely relieved by the depletion of the ORMDL proteins. Therefore our results show that the ORMDL proteins are responsible for homeostatic regulation of ceramide biosynthesis in response to exogenous ceramide. Furthermore these experiments establish that, like the yeast Orm proteins, the ORMDL proteins are negative regulators of SPT.

To ensure that the increases seen in [^{3}H]serine labeling with ORMDL knockdown were due to *de novo* ceramide biosynthesis rather than changes in serine uptake, we measured uptake of [^{3}H]serine under knockdown and C6-ceramide treatment conditions in HeLa cells. In neither case was uptake of [^{3}H]serine markedly affected, with the exception of a slight decrease in [^{3}H]serine uptake noted in C6-ceramide-treated cultures (Fig. 1*D*). This reduction was the same for both control and ORMDL-depleted cells.

In contrast to the ORMDL proteins, siRNA-mediated knockdown of SMSr did not result in increased [^{3}H]serine labeling of ceramides under normal growth conditions in any of three cell types tested, nor did SMSr knockdown affect the response of cells to C6-ceramide treatment (Fig. 1, *A–C*). However, it is clear that depletion of SMSr elevates steady-state levels of ceramide (12). We conclude that the major site of SMSr regulation of ceramide is not at the level of biosynthesis, but rather by regulating one or more of the upstream metabolic processes that determine ceramide levels. This could in principle include ceramidases or the conventional sphingomyelin synthases. When cells were transfected with a combination of siRNA oligonucleotides targeting both SMSr and all three ORMDL isoforms, we did not see an additive increase in the amount of labeled ceramide above that seen with ORMDL knockdowns alone (Fig. 1, *A–C*). This suggests that the ORMDL proteins are the primary regulators of the biosynthetic homeostatic response.

The Three ORMDL Isoforms Are Redundant in Controlling the Response of HeLa cells to C6-ceramide—Breslow *et al.* (6) noted that knockdown of all three ORMDL proteins raised steady-state ceramide levels, whereas knockdown of ORMDL3 alone had no effect. We therefore determined the contribution of the individual ORMDL isoforms to regulation of ceramide biosynthesis. HeLa cells were transfected with either scrambled siRNA oligonucleotides as a control or gene-specific siRNAs targeting all three *ORMDL* isoforms at once, paired combina-

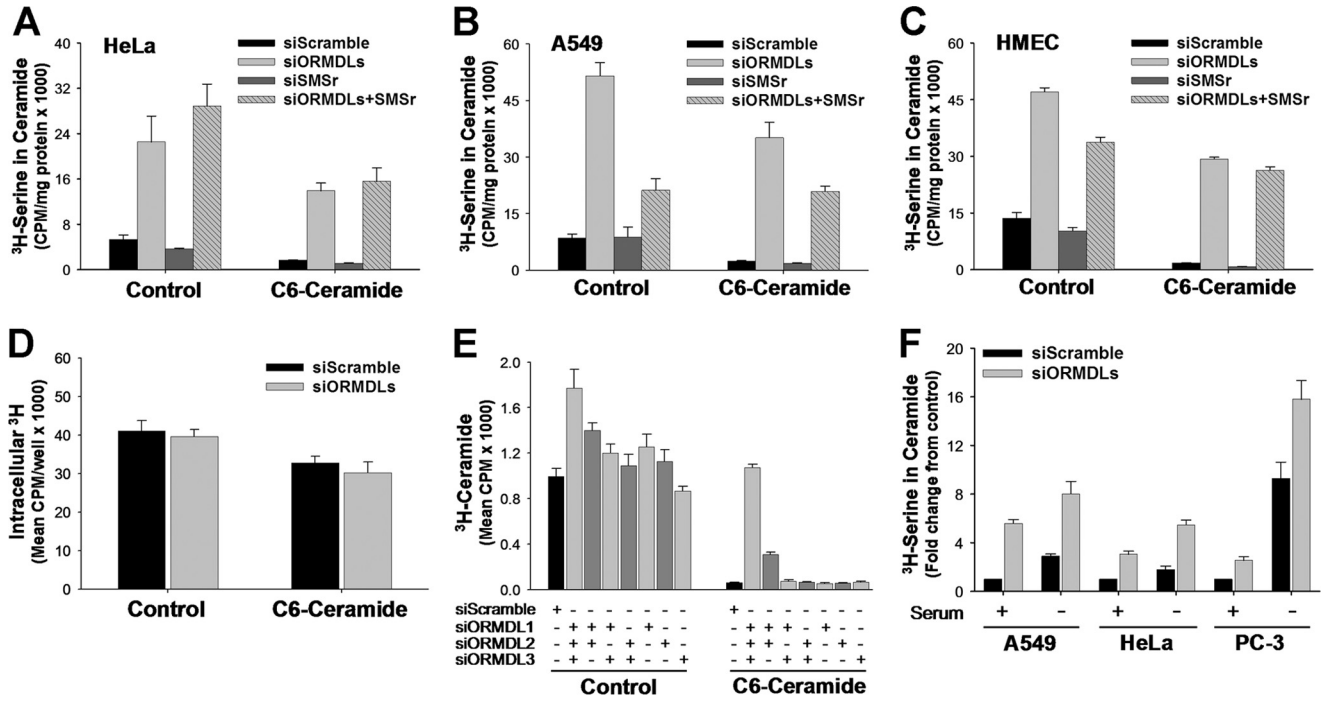

FIGURE 1. **Mammalian ORMDL proteins mediate the response of cells to C6-ceramide.** *A–C*, effect of ORMDL and SMSr depletion on ceramide biosynthesis. [^{3}H]Serine incorporation into ceramide was measured in HeLa (*A*), A549 (*B*) and HMEC (*C*) cells 48 h after transfection with either scrambled siRNA oligonucleotides or gene-specific siRNAs for all three *ORMDL* isoforms, *SMSr* alone, or a combination of siRNAs targeting *ORMDL1/2/3* and *SMSr*. After transfection, cells were treated with 10 μM C6-ceramide for 2 h and then labeled with [^{3}H]serine for 1 h. Cells were then harvested, and lipids were extracted as detailed under "Experimental Procedures." Data are reported as mean cpm/mg of protein $\pm$ S.D. ($n = 5$) and are representative of at least three independent experiments for each cell type tested. *D*, uptake of [^{3}H]serine. HeLa cells were transfected with siRNA oligonucleotides as described above, treated with 10 μM C6-ceramide for 2 h, and then labeled with [^{3}H]serine for 1 h. Cells were then harvested with trypsin, and total intracellular ^{3}H was quantitated on 10% of cells using liquid scintillation counting. Data are reported as mean cpm/well $\pm$ S.D. ($n = 6$) and are representative of three independent experiments. *E*, combinatorial knockdown of ORMDL isoforms. HeLa cells were transfected with either scrambled siRNA oligonucleotides as a control or gene-specific siRNAs for: (i) all three *ORMDL* isoforms at once; (ii) paired combinations targeting two *ORMDL* isoforms at the same time; or (iii) each *ORMDL* isoform singly. 48 h after transfection, cells were treated with 10 μM C6-ceramide for 2 h and then labeled with [^{3}H]serine for 1 h. Cells were then harvested, and lipids were extracted as detailed under "Experimental Procedures." Data are reported as mean cpm/well $\pm$ S.D. ($n = 5$) and are representative of at least three independent experiments. *F*, serum-responsive ceramide biosynthesis. Monolayers of cells were transfected with either scrambled siRNA or siRNA targeting all three *ORMDL* isoforms. 24 h after transfection, culture medium was replaced with serum-free medium containing 0.1% fatty-acid free BSA. Serum starvation was carried out for ~30 h, after which time the medium was removed and the cells were labeled with [^{3}H]serine for 1 h. Lipids were extracted, and [^{3}H]serine incorporation into ceramide was measured as detailed under "Experimental Procedures." Data are reported as -fold change $\pm$ S.D. ($n = 5$) from cells transfected with scrambled siRNA in the presence of serum for A549, HeLa, and PC-3 cells (*Control*, mean [^{3}H]serine incorporation was 1420 cpm/mg of protein for A549, 6392 cpm/mg of protein for HeLa, and 176 cpm/mg of protein for PC-3). Results for all cell lines are representative of at least three independent experiments.

tions targeting two *ORMDL* isoforms at the same time, or siRNAs for each *ORMDL* isoform singly. 48 h after transfection, cells were treated with C6-ceramide and labeled with [^{3}H]serine as described above. As depicted in Fig. 1*E*, all three isoforms must be depleted to reverse the inhibition of ceramide biosynthesis resulting from the treatment of cells with C6-ceramide. A similar effect is noted for the elevated ceramide biosynthesis in cells under normal growth conditions (Fig. 1*E*, controls). ORMDL3 may have a somewhat reduced role as depletion of ORMDL1 and -2 alone slightly reverses the C6-ceramide response. It is unclear what separate roles the three isoforms play. We have found that expression levels of the three isoforms are similar across a wide array of cell types (data not shown). Additionally, they are very highly conserved, exhibiting 81–84% identity at the amino acid level with most of the substitutions in the putative membrane spanning portions of the proteins (16). Otherwise, the three ORMDL isoforms have identical lengths, are similarly distributed in both adult and embryonic tissues, and are all localized to the endoplasmic reticulum (16). The distinction may be at the level of regulation of expression. ORMDL3 expression levels are regulated in human lung fibroblasts by poly(I:C) (17). Further, there is an association of elevated risk for asthma with genetic polymorphisms upstream of the ORMDL3 gene that affect ORMDL3 gene expression (11). Additional study will be required to establish the conditions that differentially regulate the three ORMDL isoforms.

ORMDL Proteins and Additional Mechanisms Play a Role in the Regulation of Ceramide Synthesis following Serum Starvation—Serum starvation significantly enhances ceramide biosynthesis in several cell types tested (Fig. 1*F*). Serum-responsive ceramide synthesis is further stimulated by depletion of ORMDL proteins. If ORMDL proteins are the sole regulators of ceramide biosynthesis, then their depletion should result in maximally elevated ceramide biosynthesis, and that elevated synthesis should be independent of growth conditions. However, in HeLa and PC-3 cells, elevation of ceramide biosynthesis by ORMDL knockdown is considerably higher in combination with serum deprivation than with ORMDL depletion alone. This indicates that the elevation in ceramide biosynthesis seen with serum deprivation is not solely due to removing the negative regulation of the ORMDL proteins, but is also the result of

an additional regulatory mechanism. The metabolic action of sphingosine kinase is one possible regulatory process. Our group and others have suggested that sphingosine kinase, by consuming dihydrosphingosine, a precursor in the ceramide biosynthetic pathway, can regulate ceramide biosynthesis (13, 18, 19). Alternatively, regulatory subunits of SPT (3–5) may be involved in the serum-dependent regulation of ceramide biosynthesis.

ORMDL Proteins Mediate the Response of SPT to Exogenous Ceramide in Permeabilized Cells—We expect that the ORMDL proteins would regulate SPT activity as yeast Orm proteins interact with SPT subunits (6). Moreover it is generally accepted that SPT is rate-limiting for sphingolipid synthesis. However, in the yeast studies, SPT activity was not directly measured, but rather the effect on SPT was inferred from effects of Orm depletion on steady-state sphingolipid levels and by measuring cell viability under diagnostic conditions (6). Direct measurement of the effect of the ORMDLs on SPT activity was complicated by our inability to detect changes in SPT activity in crude membrane fractions in response to C6-ceramide treatment of cells (data not shown). This suggests that an element of the regulatory mechanism is lost on preparation of membranes. To circumvent this difficulty, we used permeabilized cells to directly demonstrate that the ORMDL proteins regulate SPT activity (Fig. 2*A*). Cells were first treated with either control oligonucleotides or siRNA oligonucleotides directed against all three ORMDL isoforms. The cells were then permeabilized and treated with or without C6-ceramide for 30 min. The permeabilized and treated cells were then labeled with [^{3}H]serine and analyzed for incorporation of [^{3}H]serine into total sphingolipids (rather than ceramide alone) as a measure of SPT activity. A significant finding here is that the C6-ceramide regulation of the ORMDL proteins can be recapitulated in permeabilized cells. C6-ceramide inhibition of SPT activity involves sensing the elevation of ceramide levels, transmission of that information to the ORMDL proteins, and an interaction of the ORMDL proteins with SPT to inhibit enzymatic activity. Our ability to reproduce all of these steps in permeabilized cells indicates that diffusible cytosolic components are not involved. Apparently, the ceramide regulatory mechanism is contained within the ER membrane, the site of SPT and the ORMDLs (16, 20). The yeast Orm proteins are regulated by phosphorylation mediated by Ypk1, a soluble kinase that associates with the plasma membrane under certain conditions (6, 9, 10). It is notable that the mammalian isoforms are N-terminally truncated relative to the yeast proteins and therefore lack the three serine residues that are phosphorylated by Ypk1 in the yeast proteins (6, 16), nor do the ORMDL proteins contain canonical phosphorylation sites for the mammalian Ypk1 homologue, the serum- and glucocorticoid-induced kinase (SGK). The ORMDL proteins may be phosphorylated by a different, ceramide-sensitive kinase. Alternatively, ORMDL proteins may be regulated by a ceramide-sensitive protein-protein interaction or by an allosteric effect on the ORMDL proteins themselves. It is notable that the ORMDL proteins have four predicted membrane-spanning elements, which could potentially form ceramide-sensing domains similar to the sterol sensing of domains found in elements of the HMG-CoA reductase regulatory mechanism.

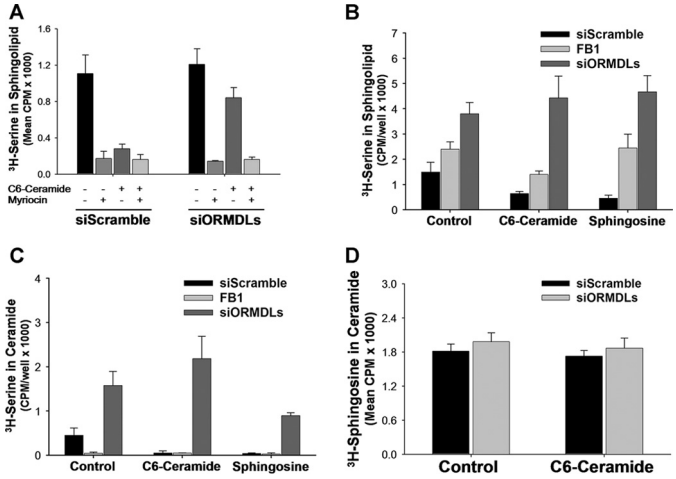

FIGURE 2. **ORMDL proteins regulate SPT in permeabilized and intact cells, but do not regulate ceramide synthases.** C6-ceramide and sphingosine require metabolism by ceramide synthases to activate ORMDL inhibition of SPT. *A*, SPT activity in permeabilized cells. HeLa cell monolayers were treated with either scrambled siRNA or siRNA targeting all three *ORMDL* isoforms. 24 h after transfection, cells were permeabilized with digitonin and preincubated in the presence or absence of 20 μM C6-ceramide for 30 min as described under "Experimental Procedures." Buffer containing [^{3}H]serine and palmitoyl-CoA was added, and the incubation was continued for an additional 60 min. Selected wells were treated with 1 μM myriocin during both incubation periods. Sphingolipids were then extracted, and incorporation of [^{3}H]serine into total sphingolipids was determined by liquid scintillation counting. Each point represents the mean ± S.D. of eight replicates, with the exception of myriocin-treated wells, which utilized four replicates. A result representative of four independent experiments is presented. *B*, effect of blocking ceramide synthase activity on C6-ceramide and sphingosine inhibition of total sphingolipid synthesis. Intact HeLa cell monolayers were treated overnight with FB1 where indicated, treated with either 10 μM C6-ceramide or 10 μM sphingosine for 2 h, and then labeled with [^{3}H]serine to measure *de novo* biosynthesis of total sphingolipid as described under "Experimental Procedures." Shown is myriocin-inhibitable incorporation of [^{3}H]serine into total sphingolipids. *C*, C6-ceramide and sphingosine inhibition of ceramide synthesis. Lipid extracts generated as described for *panel A* were analyzed for [^{3}H]serine incorporation into ceramide by thin layer chromatography. Shown is myriocin-inhibitable incorporation of [^{3}H]serine into ceramide. *D*, ceramide synthase activity in intact cells. HeLa cells were transfected with siRNA oligonucleotides as described above, treated with 10 μM C6-ceramide for 2 h, and then labeled with D-[erythro-3-^{3}H]sphingosine for 30 min. Cells were then harvested, and lipids were extracted as detailed under "Experimental Procedures." Data in *panels B–D* are reported as mean cpm/well ± S.D. (n = 3–6) and are representative of at least three independent experiments.

ORMDL-mediated Feedback Inhibition of Sphingolipid Synthesis by Sphingosine or C6-ceramide Requires Ceramide Synthase Activity—C6-ceramide is known to be remodeled to native ceramide species by the sequential action of ceramidase and ceramide synthase (21–24). This remodeling is required, for example, to mediate the growth inhibitory properties of C6-ceramide (22). Therefore it is not clear whether the ORMDL-dependent inhibition of SPT is in response to C6-ceramide itself, to native ceramides generated by remodeling, or to the sphingosine liberated from C6-ceramide by ceramidases. Exogenous sphingosine is also known to inhibit *de novo* sphingolipid biosynthesis (15, 25). As with C6-ceramide, it is not known whether it is sphingosine itself that mediates this response, or whether sphingosine must be further metabolized to ceramides. To establish which of these lipids mediates feedback inhibition of SPT, we incubated cells in the presence of either C6-ceramide (as before) or sphingosine in the presence

or absence of the ceramide synthase inhibitor Fumonisin B1 (FB1). Because FB1 will inhibit *de novo* ceramide biosynthesis, we measured total sphingolipid biosynthesis (Fig. 2*B*) as a measure of SPT activity. Ceramide biosynthesis was also measured (Fig. 2*C*). As expected, both C6-ceramide and sphingosine were inhibitors of total *de novo* sphingolipid and ceramide biosynthesis, and this inhibition was reversed by ORMDL depletion (Fig. 2, *B* and *C*). To determine whether further metabolism of C6-ceramide and sphingosine was required for their inhibitory effects, we pretreated cells with the ceramide synthase inhibitor fumonisin B1. This treatment completely inhibited ceramide biosynthesis, as expected (Fig. 2*C*). FB1 treatment completely reversed the inhibitory effect of sphingosine treatment and largely reversed the effect of C6-ceramide treatment. This indicates that ceramides or higher order sphingolipids (sphingomyelin or glycosphingolipids) are the sphingolipid components that activate the ORMDL regulatory system. We consistently observe that FB1 does not as completely reverse C6-ceramide inhibition when compared with the reversal of sphingosine inhibition. C6-ceramide may weakly activate this system by itself. C6-ceramide is also known to activate sphingomyelinase to indirectly generate increased ceramide levels (23). Ceramide levels elevated by that mechanism might also feed back to the ORMDL regulatory mechanism.

ORMDL Proteins Do Not Regulate Ceramide Synthases—We considered that the ORMDL proteins might control ceramide biosynthesis at the level of ceramide synthases in addition to their regulation of SPT. HeLa cells were transfected with siRNAs targeting all three ORMDL isoforms and treated with C6-ceramide exactly as described above. Following C6 treatment, cells were labeled with D-[erythro-3-^{3}H]sphingosine (rather than [^{3}H]serine) for 30 min. Lipids were harvested, and ceramide was extracted. Unlike [^{3}H]serine labeling of ceramides as seen in Fig. 1 (*A*–*C*), loss of ORMDL proteins did not result in increased incorporation of D-[erythro-3-^{3}H]sphingosine into ceramide (Fig. 2*D*). This indicates that the ORMDL proteins do not regulate the ceramide synthases.

Conclusions—As highlighted by Breslow and Weissman (26), ceramide shares the property with cholesterol of being an essential cellular component that, because of deleterious effects at elevated levels, is subject to tight metabolic control. The studies presented here illustrate that the ORMDL proteins are part of a complicated network of regulatory mechanisms that accomplish this balancing act. The ORMDL proteins act as the effector of one of these mechanisms by regulating SPT activity. The nature of the ceramide sensor that regulates the ORMDL proteins in mammalian cells remains to be determined, as do the details of how ORMDL interacts with subunits of SPT to negatively regulate SPT activity. We were unable to detect any effect of SMSr on ceramide biosynthesis. However, the clear effect of SMSr on steady-state ceramide levels suggests that SMSr controls ceramide levels through upstream metabolism. In this light, the ORMDL and SMSr proteins constitute complementary regulatory systems in which the overall levels of ceramide are determined by a balance of biosynthesis, regulated by the ORMDL proteins, and upstream metabolism, determined by SMSr through phosphoethanolamine ceramide. We also note that there is an as yet undescribed mechanism that controls ceramide biosynthesis in response to serum starvation. Therefore although the ORMDL proteins constitute the major homeostatic regulators of ceramide biosynthesis, the complete mechanism of control remains to be elucidated.

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