Two Mammalian Longevity Assurance Gene (LAG1) Family Members, trh1 and trh4, Regulate Dihydroceramide Synthesis Using Different Fatty Acyl-CoA Donors

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Overexpression of upstream of growth and differentiation factor 1 (uag1), a mammalian homolog of the yeast longevity assurance gene (LAG1), selectively induces the synthesis of stearoyl-containing sphingolipids in mammalian cells (Venkataraman, K., Riebeling, C., Bodennec, J., Riezman, H., Allegood, J. C., Sullards, M. C., Merrill, A. H. Jr., and Futerman, A. H. (2002) J. Biol. Chem. 277, 35642–35649). Gene data base analysis subsequently revealed a new subfamily of proteins containing the Lag1p motif, previously characterized as translocating chain-associating membrane (TRAM) protein homologs (TRH). We now report that two additional members of this family regulate the synthesis of (dihydro)ceramides with specific fatty acid(s) when overexpressed in human embryonic kidney 293T cells. TRH1 or TRH4-overexpression elevated [3H](dihydro)ceramide synthesis from L-[3-3H]serine and the increase was not blocked by the (dihydro)ceramide synthase inhibitor, fumonisin B1 (FB1). Analysis of sphingolipids by liquid chromatography-electrospray tandem mass spectrometry revealed that TRH4 overexpression elevated mainly palmitic acid-containing sphingolipids whereas TRH1 overexpression increased mainly stearic acid and arachidic acid, which in both cases were further elevated upon incubation with FB1. A similar fatty acid specificity was obtained upon analysis of (dihydro)ceramide synthase activity in vitro using various fatty acyl-CoA substrates, although in a FB1-sensitive manner. Moreover, in homogenates from TRH4-overexpressing cells, sphinganine, rather than sphingosine was the preferred substrate, whereas no preference was seen in homogenates from TRH1-overexpressing cells. These findings lend support to our hypothesis (Venkataraman, K., and Futerman, A. H. (2002) FEBS Lett. 528, 3–4) that Lag1p family members regulate (dihydro)ceramide synthases responsible for production of sphingolipids containing different fatty acids.

Ceramide is an important intracellular second messenger involved in a number of regulatory processes (1). Intracellular ceramide levels can be altered by a variety of mechanisms, including de novo synthesis, which can impact upon cell growth, regulation, differentiation, and death (2–7). A key enzyme in the de novo pathway is sphinganine-N-acyl transferase (dihydroceramide synthase). Significant steps toward determining the modes of regulation of this enzyme were achieved by the demonstration that the yeast proteins, Lag1p and Lac1p, are required for the synthesis of very long chain (C26) ceramides in yeast (8), and that Alternaria stem canker locus-1 (asc1), a member of the longevity assurance gene (lag) family, mediates resistance to fumonisins B1 (FB1), a dihydroceramide synthase inhibitor (9–11), in tomato (12, 13). We subsequently demonstrated that overexpression of a mammalian homolog of these proteins, upstream of growth and differentiation factor 1 (UOG1), conferred FB1-resistance in mammalian cells, and unexpectedly, specifically regulated the synthesis of stearoyl-containing sphingolipids (SLs) (14). Data base analyses then revealed a family of Lag1p motif-containing proteins (15–17), UOG1 was classified with fungal and plant Lag1p homologs, and a new branch of the subfamily containing a homology-like domain at the N terminus was revealed in animals. Five mammalian proteins, originally characterized as translocating chain-associating membrane (TRAM) protein homologs (TRH), were detected, and we suggested that different members of this family might regulate the N-acylation of sphingoid long chain bases using different fatty acyl CoA species (16).

We now demonstrate that overexpression of TRH1 and TRH4 in mammalian cells increases (dihydro)ceramide synthesis and confers FB1 resistance in vivo but not in vitro. Moreover, TRH1-overexpressing cells are preferentially enriched in SLs containing stearic and arachidic acid, whereas SLs synthesized by TRH4-overexpressing cells are preferentially enriched in palmitic acid. These data support our suggestion (16) that different Lag1p family members regulate (dihydro)ceramide synthesis utilizing different fatty acyl-CoA species.

EXPERIMENTAL PROCEDURES

Materials—L-[3-3H]serine (specific activity of 26 or 34 Ci/mmol) and 1-[14C]-stearoyl-CoA (specific activity of 55 mCi/mmol) were from Amersham Biosciences, 1-[14C]-palmitoyl-CoA (specific activity of 54 mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO) and

1 The abbreviations used are: lag, longevity assurance gene; ER, endoplasmic reticulum; ESI-MS/MS, electrospray ionization-tandem mass spectrometry; FB1, fumonisin B1; GlcCer, glucosylceramide; GSL, glycosphingolipid; HA, hemagglutinin; PDI, protein-disulfide isomerase; SL, sphingolipid; SM, sphingomyelin; TRAM, translocating chain-associating membrane; TRH, TRAM homologs; uag, upstream of growth and differentiation factor.
Regulation of Dihydroceramide Synthesis by TRH1 and TRH4

Table 1
Primers and PCR conditions used in this study

| Gene       | Sequences                                      | Tm [°C] | Cycles |
|------------|------------------------------------------------|---------|--------|
| trh1       | 5'-AAAAGCTTATGCTGTCAGCTGTTAGTAGTA             | 52      | 35     |
| trh1 (N-terminal tag) | 5'-AACAGCTGCTGCTGTTAGTAGTA             | 52      | 35     |
| trh1 (C-terminal tag) | 5'-AACAGCTGCTGCTGTTAGTAGTA             | 60      | 35     |
| trh4       | 5'-AAATCTGACATTGCAACAGGATTGTTAGAT            | 59      | 35     |
| trh4 (N-terminal tag) | 5'-AAATCTGACATTGCAACAGGATTGTTAGAT            | 57      | 35     |
| trh4 (C-terminal tag) | 5'-AAATCTGACATTGCAACAGGATTGTTAGAT            | 72      | 35     |

* Restriction sites used for cloning are underlined.

D-erythro-[4,5-3H]sphinganine was synthesized as described (18, 19). Ceramide, FB, glucosyleramides (GlcCer), galactosyleramides, sphingomyelin (SM) palmitoyl-CoA, stearoyl-CoA, arachidonyl-CoA, defatted bovine serum albumin, phenethylsulfonyl fluoride, leupeptin, antipain, and aprotinin were from Sigma. Behenoyl-CoA, lignoceroyl-CoA, bovine serum albumin, phenylmethylsulfonyl fluoride, leupeptin, anti-HA monoclonal antibody (clone 3F10) was from Roche Applied Biosystems (Alabaster, AL). Reverse phase RP-18 columns were from Supelco (Bellefonte, PA). pcDNA3.0 was from Invitrogen (Carlsbad, CA). A pGEMT cloning kit, TaqDNA polymerase, and Pfu DNA polymerase were from Promega Corporation (Madison, WI). Restriction enzymes and DNA modifying enzymes were either from MBIB Ferments (Vilnius, Lithuania) or from New England Biolabs (Beverly, MA). A rat anti-HA monoclonal antibody (clone 3F10) was from Roche Applied Science, a rabbit anti-protein-disulfide isomerase (PDI) antibody was from Stressgen (Victoria, Canada), and Mitotracker® Deep Red was from Molecular Probes (Eugene, OR). Silica gel 60 TLC plates were from Merck. All solvents were of analytical grade and were purchased from Biobal (Jerusalem, Israel). Oligonucleotides were synthesized by the Weizmann Institute Oligonucleotide and Peptide Synthesis facility.

Cloning of trh1 and trh4—Mouse expression sequence tag clones, L.M.A.G.E. ID 1210358 and 4288182, were obtained from the American Tissue Culture Collection, (Manassas, VA). Sequencing by the DNA sequencing unit of the Weizmann Institute of Science revealed that the clones contained the full-length cDNA sequences of the mouse trh1 and trh4 genes (GenBank™ accession numbers Y10928531 and AK010241), respectively. Fragments containing the cDNA were amplified using Pfu polymerase with primers containing unique restriction sites (Table 1), the PCR products were cloned using the pGEMT vector, and then subcloned using the introduced restriction sites into pcDNA3.0 or pcDNA3.0HA. The coding sequences of all constructs were confirmed by nucleotide sequencing.

Cell Culture and Transfection— COS-7 cells grown on glass cover slips, and human embryonic kidney 293T cells and HeLa cells grown in tissue culture flasks, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Transfections were performed by the calcium phosphate method (20) using 1 μg of plasmid per cm² of culture dish, yielding a transfection efficiency of ~10% for COS-7 cells and ~95% for 293T and HeLa cells as determined by transfection with a green fluorescent protein-encoding vector.

Metabolic Labeling—Twenty-four hours after transfection, 293T cells were incubated with 10 μCi/ml L-[3-3H]serine, with or without 20 μM FB, for a further 24 h. Cells were removed from the culture dishes by scraping into methanol using a rubber policeman, and lipids extracted (21), subjected to alkaline hydrolysis in chloroform, 0.6 n NaOH in methanol (1:1) (room temperature, 2 h) to degrade glycerolipids (22), split into three aliquots, and mixed with authentic standards (see below). [3H]SLs were separated by TLC using the following developing solvents: (i) [3H](dihydro)ceramide was separated using chloroform/methanol/MeOH/CH3CN (95:5:8:2; v/v); (ii) [3H]GlcCer and [3H]galactosyleramide were separated on a sodium borate-coated TLC plate using chloroform/methanol/water (50:40:10, v/v/v); (iii) neutral [3H]SLs were separated on two-dimensional TLC using tetrahydronfuran/acetonitrile/methanol/water (50:20:40:6, v/v/v/v) and chloroform/acetonitrile/methanol/acetic acid/water (50:20:10:15:5, v/v/v/v/v); (iv) for analysis of acidic [3H]SLs, the aqueous phase of the lipid extract (21) was loaded onto Supelco RP-18 disposable cartridges (23) and washed with water. Lipids were eluted using chloroform/methanol (1:1; v/v) and separated by TLC using chloroform/methanol/9.8 mM CaCl2 (60:35:8, v/v/v). Lipids were visualized using I2 and recovered from the TLC plates by scraping the silica directly into scintillation vials. 1 ml of methanol and 5 ml of Optima Gold scintillation fluid (Packard, Dovers Grove, IL) were added to each vial for scintillation counting, and radioactivity determined using a Packard 2100 beta radiospectrometer equipped with the Transformed Spectral Index of the External Standard/Automatic Efficiency Control (TSE/AEC) program for quench correction.

Electrospray Tandem Mass Spectrometry—293T cells were transfected, and after 24 h harvested by trypsinization, and collected by centrifugation. Cell pellets were washed twice with ice-cold phosphate-buffered saline and lyophilized. SLs were extracted and analyzed by liquid chromatography, electrospray tandem mass spectrometry (ESI-MS/MS) on a Finnigan API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray source as described previously (24). The orifice and ring voltages were kept low (40 V and 220 V, respectively) to prevent collisional decomposition of molecular ions prior to entry into the first quadrupole; the orifice temperature was set to 500 °C. N2 was used to collisionally induce dissociations in Q2, which was offset from Q1 by...
TABLE II

Amounts of long chain bases and SLs in trh1- and trh4-transfected cells

293T cells were transfected with pcDNA (mock), pcDNA-TRH1, or pcDNA-TRH4, and 24 h later, incubated with or without 20 μM FB1 for a further 24 h. Results are means ± S.D. for three independent experiments. Numbers in parentheses show the amounts in TRH1- or TRH4-transfected cells versus the mock-transfected cells as a percentage.

|                 | TRH1 | TRH1 + FB1 | TRH1 | TRH1 + FB1 | TRH4 | TRH4 + FB1 | TRH4 | TRH4 + FB1 |
|-----------------|------|------------|------|------------|------|------------|------|------------|
| Regulator       |       |            |      |            |      |            |      |            |
| Sphinganine      | 5.7 ± 2.6 (58) | 720 ± 60 (68) | 5.7 ± 2.6 (58) | 720 ± 60 (68) | 13 ± 5 (220) | 13 ± 5 (220) | 7 ± 1 (150) | 7 ± 1 (150) |
| Sphinganine-1-phosphate | 7.8 ± 3.0 (97) | 840 ± 100 (68) | 7.8 ± 3.0 (97) | 840 ± 100 (68) | 3.8 ± 0.7 (58) | 3.8 ± 0.7 (58) | 2.1 ± 0.5 (128) | 2.1 ± 0.5 (128) |
| Dihydroceramide  | 1.1 ± 0.5 (227) | 140 ± 20 (22) | 1.1 ± 0.5 (227) | 140 ± 20 (22) | 1.6 ± 0.8 (158) | 1.6 ± 0.8 (158) | 2.1 ± 0.5 (229) | 2.1 ± 0.5 (229) |
| Dihydroceramide Synthase Assay—293T cells were transfected with pcDNA (mock), pcDNA-TRH1, or pcDNA-TRH4 for 24 h, and then incubated for 24 h with 10 μCi/mL of [3-3H]serine in the presence or absence 20 μM FB1. Results are means ± S.D. for 3–4 independent experiments.

40–50 V. Q3 was then set to pass molecularly distinctive product ions (N ions) of m/z 264.4 and 266.4 for sphingosine- and sphinganine-containing SLs, respectively. Multiple reaction monitoring (MRM) scans were acquired by setting Q1 and Q3 to pass the precursor and product ions of the most abundant SL molecular species. For example, for the ceramides, these transitions occur at m/z 538.7/264.4, 566.5/264.4, 622.7/264.4, 648.7/264.4, 650.7/264.4, 676.5/264.4, and 678.5/264.4, which correspond to ceramides with a d18:1 sphingoid base (sphingosine) and C16:0, C18:0, C22:0, C24:1, C24:0, C26:1, and C26:0 fatty acids, respectively. Quantitation was achieved by spiking the samples prior to extraction with the C12-fatty acid homologs of ceramide, SM, and GlcCer.
tracted (25) and (dihydro)ceramide synthesis was analyzed by TLC using chloroform/methanol/2 M ammonium hydroxide (40:10:1; v/v/v) as the developing solvent. Lipids were visualized using a phosphorimager screen (Fuji, Tokyo, Japan), recovered from TLC plates by scraping the silica directly into scintillation vials and quantified by liquid scintillation counting.

Immunolocalization—Eighteen hours after transfection, COS-7 cells were incubated with MitoTracker® Deep Red (25 nM) for 15 min and chased for 3 min in fresh medium that did not contain MitoTracker® Deep Red. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (20 min, 37 °C), and permeabilized with 0.1% (v/v) Triton X-100 in phosphate-buffered saline/1% bovine serum albumin for 30 min. Primary antibodies (rabbit anti-PDI, dilution of 1:200; rat anti-HA, 1:500) were applied for 2 h at room temperature. A rhodamine-labeled goat anti-mouse secondary antibody was diluted 1:200, and a fluorescein isothiocyanate-labeled goat anti-rabbit secondary antibody was diluted 1:200. After 1 h incubation, slides were mounted in Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL) and examined by confocal laser scanning microscopy using an Olympus Fluoview FV500 imaging system. Fluorescein isothiocyanate, rhodamine fluorescence, and MitoTracker® Deep Red were viewed using an argon and two helium-neon laser with excitation wavelengths of 488, 543, and 633 nm, respectively. Images were acquired in sequential mode and analyzed using Fluoview 3 imaging software.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Mice (males, 3-month-old) were sacrificed, tissues removed, rapidly frozen in liquid N₂, and stored at −80 °C. Total RNA was isolated from whole tissues using the TRI reagent (MRC, Cincinnati, OH) and purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RT-PCR was performed as described (26) using the Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany). Primers were designed using the MacMolly Tetra program (Softgene, Berlin, Germany) and PCR cycling performed using the conditions described in Table I, which gives the primers for all six Lag1p family members (16).

RESULTS

Metabolic Labeling of trh1- and trh4-transfected Cells—To determine whether TRH1 and TRH4 affect SL synthesis, trh1 and trh4 were subcloned into a pcDNA3.0 vector and used to transiently transfect 293T cells prior to metabolic labeling for 24 h with [3H]serine, a substrate for the first enzyme of SL biosynthesis, serine palmitoyl transferase (27, 28). A 1.8-fold
and a 1.5-fold increase in \(^{3}H\) (dihydro)ceramide labeling was observed from mock-, \(trh1\)-, or \(trh4\)-transfected 293T cells, respectively, compared with mock-transfected cells (Fig. 1). FB\(_1\) inhibited (dihydro)ceramide labeling in mock-transfected cells, but \(^{3}H\) (dihydro)ceramide was 3–4-fold higher in both \(TRH1\) and \(TRH4\) overexpressing 293T cells upon FB\(_1\) treatment (Fig. 1).

A 1.8-fold increase in \(^{3}H\)GlcCer labeling was observed in \(TRH1\)-overexpressing 293T cells and a small increase in \(^{3}H\)GlcCer separation into two bands using chloroform/methanol/water (24:7:1, v/v/v) as developing solvent. The lower band, which consists of shorter acyl chain fatty acids (i.e., C16) and \(\beta\)-hydroxy fatty acids (46), was elevated 2.8-fold whereas \(^{3}H\)GlcCer in the upper band was reduced by 0.8-fold. This variation was not due to changes in levels of \(\beta\)-hydroxy fatty acids as no change in their levels could be detected by ESI-MS/MS. The increase in \(^{3}H\)GlcCer synthesis represents the mean change taking into account the different amounts of \(^{3}H\)GlcCer detected in each band.
and [3H]globoside labeling were also increased in TRH1- and to a lesser extent in TRH4-overexpressing cells, and not inhibited by FB1 (not shown). [3H]Ganglioside labeling was much lower in 293T cells than neutral [3H]GSL labeling and only moderately affected by overexpression, but also showed FB1 resistance. The distribution of [3H]dihydroceramide showed a slight preference for incorporation into neutral GSLs for TRH1-overexpressing cells and for acidic GSLs in TRH4-overexpressing cells (not shown).

SL Amounts and Fatty Acid Composition in trh1- and trh4-transfected 293T Cells—We next examined long chain base and SL amounts by ESI-MS/MS. Consistent with the metabolic labeling results (Fig. 1), a ~2-fold increase in dihydroceramide and ceramide mass was observed in trh1- and trh4-transfected cells (Table II), and ceramide monohexosides and SM were also elevated. Upon incubation of mock-transfected cells with FB1, dihydroceramide and ceramide amounts decreased to 47% and 21% of non-treated cells, whereas in TRH1- and TRH4-overexpressing cells, dihydroceramide mass was increased by 18–20-fold, and ceramide by ~2.5-fold.

In both mock- and TRH1- or TRH4-transfected cells, a large increase in sphinganine was detected, presumably due to inhibition of an endogenous FB1-sensitive dihydroceramide synthase (9), although a greater increase in sphinganine levels was seen in mock-compared with TRH1- or TRH4-transfected cells. Sphinganine 1-phosphate was only detected in FB1-treated cells, and similar to sphinganine, sphinganine 1-phosphate was lower in TRH1- and TRH4-transfected cells than in FB1-treated mock-transfected cells (Table II).

Analysis of the fatty acid composition of SLs in TRH1- and TRH4-overexpressing cells revealed major changes compared with mock-transfected cells. TRH1-overexpressing cells exhibited a strong increase in N-linked stearoyl- (C18), arachidoyl- (C20), and to a lesser extent behenoyl (C22) ceramides, mono- and dihexosyl ceramides, and SM (Fig. 2). In TRH4-overexpressing cells, N-linked palmitoyl (C16) SLs were strongly increased (Fig. 2), and there were no notable differences in other fatty acids.

Changes were also observed in the ratio of sphinganine-versus sphingosine-containing SLs. C16-ceramide and C16-dihydroceramide were increased to a similar extent in TRH4-overexpressing cells, and after FB1 treatment, C16-dihydroceramide was ~2-fold higher than C16-ceramide. Likewise, after FB1 treatment, C18- and C20-dihydroceramide were significantly higher than C18- and C20-ceramide in TRH1-overexpressing cells. In contrast, complex SLs (mono- and dihexosyl ceramide and SM) contained mainly ceramides rather than dihydroceramides with or without FB1 treatment (Fig. 2).

Analysis of (Dihydro)ceramide Synthase in Vitro—A similar selectivity toward fatty acids was obtained upon analysis of dihydroceramide synthase activity in vitro (18, 19). Using the same concentration of each fatty acyl-CoA (50 μM) in the reaction mixture, and using [4,5-3H]sphinganine, dihydroceramide synthase activity was increased 13- and 16-fold in homogenates from trh1-transfected cells using either stearoyl-CoA or arachidoyl-CoA, respectively, but no increases were apparent using other fatty acyl-CoAs (Fig. 3). In homogenates from trh4-transfected cells, a 31- and 17-fold increase in dihydroceramide synthase activity was observed in trh1-transfected cells using either stearoyl-CoA or arachidoyl-CoA, respectively, but no increases were apparent using other fatty acyl-CoAs. Thus, the specificity of the dihydroceramide synthase reaction in vitro is entirely consistent with the data obtained by ESI-MS/MS on the fatty acid composition of dihydroceramide.

In vitro assays using 1-[14C]palmitoyl-CoA (for TRH4) or 1-[14C]stearoyl-CoA (for TRH1) revealed similar levels of incorporation of sphingosine and sphinganine into (dihydro)ceramide in homogenates from TRH1-overexpressing cells, and an ~2-fold greater incorporation of sphinganine compared with sphingosine in homogenates from TRH4-overexpressing cells (Fig. 4A), suggesting that both gene products may be, or may modulate (dihydro)ceramide synthases with different preferences for long chain bases. The in vitro (dihydro)ceramide

Fig. 6. Intracellular localization of TRH1 and TRH4 in COS-7 cells. The localization of TRH1 and TRH4, both HA-tagged at the C terminus (TRH1-HA and TRH4-HA) was compared with the ER marker, PDI, and to the mitochondria-specific dye, MitoTracker®. The merged view is shown in pseudo-color with anti-HA in red, anti-PDI in blue, and MitoTracker® in green. Areas of co-localization of anti-HA with anti-PDI are in pink, anti-HA with MitoTracker in yellow, and anti-PDI with MitoTracker® in white. Bar, 10 μm.
synthase activity of both TRH1- and TRH4-overexpressing cells was FB₃ sensitive in vitro (Fig. 4), in contrast to the FB₁ resistance observed in intact cells.

**mRNA Expression of Lag1p Homologs in Mouse Tissues—**

Analysis of the tissue distribution of trh1 and trh4 by semiquantitative RT-PCR revealed that trh1 and trh4 are ubiquitously expressed in the tissues investigated, but both show low expression in liver and trh1 is found at high levels in skin (Fig. 5). This distribution is unlike that of uog1 (14), which, as previously reported (15), is found mainly in brain and muscle and also in heart, lung and skin (Fig. 5). We also compared the tissue distribution of trh1 and trh4 with the three other Lag1p homologs, trh3, trh3-like (t3l), and trh1-like (t1l) (16). Trh3 is ubiquitously expressed, as reported (29), with expression highest in liver and intestine and low in spleen and skin, trh1-like (t1l) is also ubiquitously expressed with high expression in intestine and low expression in spleen and muscle, and trh3-like (t3l) is found at high levels in skin (Fig. 5).

**Subcellular Localization of TRH1 and TRH4—** Finally, we determined the subcellular localization of overexpressed TRH1 and TRH4 by subcloning trh1 and trh4 into pcDNA3HA vectors to give constructs expressing TRH1 and TRH4 with HA tags at the N (not shown) or C terminus (TRH1-HA and TRH4-HA). Irrespective of the position of the HA tag, TRH1 and TRH4 both co-localized with an ER marker, PDI, in HeLa and 293T cells (not shown), and in COS-7 cells, and did not show any colocalization with the mitochondrial specific dye, MitoTracker® (Fig. 6). Thus, TRH1 and TRH4 are localized to the ER, as is UOG1, although unlike UOG1, neither contain a known ER retrieval motif (30).

**DISCUSSION**

A major finding of this study is that two additional Lag1p motif-containing proteins, TRH1 and TRH4, regulate the formation of distinct molecular subspecies of (dihydro)ceramides, apparently through selective utilization of fatty acyl-CoAs. Previous studies in yeast (8, 31) have shown that the Lag1p motif-containing-proteins, Lag1p and Lac1p, utilize C26-fatty acids, and the mammalian homolog, UOG1 (14), utilizes C18-fatty acids for (dihydro)ceramide synthesis. The current study extends the number of genes that regulate dihydroceramide synthesis in mammalian cells, and increases the likelihood that other mammalian Lag1p family members (16, 17) also regulate (dihydro)ceramide synthesis with a high degree of fatty acid specificity. Interestingly, Saccharomyces cerevisiae SLs contain mainly one kind of N-linked fatty acid (namely C26) (32, 33), and only two Lag1p family members (Lag1p/Lac1p) have been found to date in yeast. A much wider fatty acid distribution is found in SLs in mammalian cells where at least six Lag1p motif-containing-proteins exist (16). Although it is not possible at this stage to predict whether all members of the mammalian gene family affect the synthesis of (dihydro)ceramides with one (or a few) fatty acids, our data suggest a highly regulated mechanism of controlling N-acylation of sphingoid bases using different fatty acyl-CoA donors.

Like most enzymes in the SL biosynthetic pathway, (dihydro)ceramide synthase has not been isolated and purified. Thus, we cannot determine whether the Lag1p family members are bone fide (dihydro)ceramide synthases or modulators of the activity of endogenous (dihydro)ceramide synthase(s). All of the Lag1p family members studied to date are localized to the ER, where (dihydro)ceramide synthase activity has previously been localized (19, 34). In a recent study, (dihydro)ceramide synthase was partially purified from bovine liver microsomes, revealing a complex of Mₛ ~333 kDa, which appeared to consist of 47 kDa subunits (35), which matches the predicted Mₛ of TRH1 (46 kDa) and TRH4 (48 kDa). Although the fatty acyl specificity of this complex has not been determined, earlier studies demonstrated that (dihydro)ceramide synthase in microsomes exhibited a preference for stearoyl-CoA > lignoceryl-CoA > palmitoyl-CoA > oleyl-CoA at a ratio of ~60:12:3:1 (36). Our data raise the possibility that different Lag1p family members are differentially expressed (15, 29), and/or differentially regulated in different tissues under different physiological conditions, and may become associated with a (dihydro)ceramide synthase complex so as to regulate (dihydro)ceramide levels containing different fatty acids. Interestingly, a recent study (37) has shown that expression of human Lag1p homologs in yeast lag1Δ lac1Δ double mutants restores acyl-CoA-dependent ceramide and SL biosynthesis, although the clones examined predominantly used C24 or C26 fatty acids. The precise mechanism by which Lag1p family members regulate (dihydro)ceramide synthesis will only be determined once they have been purified and their properties compared with endogenous, FB₁-sensitive (dihydro)ceramide synthase activities.

It is not clear why Lag1p family members are sensitive to inhibition by FB₁ when assayed in vitro but resistant to FB₁ in intact mammalian cells. One possibility is that the overexpressed protein is activated, either directly or indirectly, by a metabolite that accumulates upon FB₁-treatment, and we are currently exploring this possibility. Moreover, different cell types respond differently to FB₁ inhibition even when measured in vivo (38, 39). Thus, levels of elevation of [³H](dihydro)-ceramide and [²H]SL labeling were lower in TRH1- and TRH4-overexpressing HeLa cells after FB₁ treatment than in TRH1- and TRH4-overexpressing 293T cells, although still elevated compared with FB₁-treated mock-transfected HeLa cells. In addition, different cell types could vary in sensitivity to FB₁ inhibition due to differential expression of Lag1p family members. Thus, although trh1 and trh4 have similar tissue distributions, at least at the mRNA level, other Lag1p homologs have a more restricted tissue distribution.

Another intriguing issue concerns the possible use of different sphingoid long chain bases for SL synthesis. Thus, the (dihydro)ceramide synthase activity from TRH1-overexpressing cells was similar using sphingosine and sphinganine as substrates. TRH1-overexpressing cells exhibited a ~2-fold higher activity with sphinganine compared with sphingosine when assayed in vitro. By this criterion, TRH1 resembles a partially purified (dihydro)ceramide synthase from a mitochondria-enriched fraction (40), and a (dihydro)ceramide synthase activity in murine cerebellar microsomes (38), which exhibit a similar maximal activity using sphinganine or sphingosine (41). However, on the basis of Vₘₐₓ/Kₘₐₓ, the partially purified enzyme from mitochondria had a slight preference for sphingosine and the cerebellar microsomes showed a ~3-fold difference in Vₘₐₓ/Kₘₐₓ for sphinganine versus sphingosine (41). The preference toward sphinganine of TRH4 was somewhat reflected in the elevated dihydroceramide backbones of SLs in these cells (especially in the presence of FB₁); the increase in ceramides as well as dihydroceramides in intact cells probably reflects the ability of dihydroceramides to undergo desaturation (42, 43). Nonetheless, the existence of synthases that favor dihydroceramide formation has a potential physiological significance since ceramides, but not dihydroceramides, participate in various signaling pathways (44, 45).

In conclusion, our data lend support to our hypothesis (16) that Lag1p family members regulate (dihydro)ceramide synthase activity in vitro. Further studies are needed to determine whether the two Lag1p family members are bone fide (dihydro)ceramide synthases or modulators of the activity of endogenous (dihydro)ceramide synthase(s).

³ Western blot analysis revealed that the Mₛ of HA-tagged-TRH1 and -TRH4 is consistent with the predicted Mₛ (data now shown).
⁴ C. Riebeling and A. H. Futterman, unpublished observations.
thases responsible for production of SLs containing different fatty acids and possibly different sphingoid bases.

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