

ELOVL1 production of C24 acyl-CoAs is linked to C24 sphingolipid synthesis

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Very long-chain fatty acids (VLCFAs) exert a variety of cellular functions and are associated with numerous diseases. However, the precise pathway behind their elongation has remained elusive. Moreover, few regulatory mechanisms for VLCFAs synthesis have been identified. Elongases catalyze the first of four steps in the VLCFA elongation cycle; mammals have seven elongases (ELOVL1–7). In the present study, we determined the precise substrate specificities of all the ELOVLs by in vitro analyses. Particularly notable was the high activity exhibited by ELOVL1 toward saturated and monounsaturated C20- and C22-CoAs, and that it was essential for the production of C24 sphingolipids, which are unique in their capacity to interdigitate within the membrane as a result of their long chain length. We further established that ELOVL1 activity is regulated with the ceramide synthase CERS2, an enzyme essential for C24 sphingolipid synthesis. This regulation may ensure that the production of C24-CoA by elongation is coordinated with its utilization. Finally, knockdown of ELOVL1 caused a reduction in the activity of the Src kinase LYN, confirming that C24-sphingolipids are particularly important in membrane microdomain function.

acyl-CoA | lipid metabolism | monounsaturated fatty acid | polyunsaturated fatty acid | saturated fatty acid

Lipid metabolism is closely related to metabolic syndrome and lifestyle-related diseases including hyperlipidemia, obesity, and arteriosclerosis. Fatty acid (FA) species found in cells and in plasma lipids, such as triglycerides and cholesterol esters, are mainly C16 (C16:0, palmitic acid) and C18 (C18:1, oleic acid; C18:2, linoleic acid) long-chain FAs (LCFAs) (1). Less common FAs having longer chain length and/or a higher degree of unsaturation exhibit completely different, often beneficial, physiological and pathological properties from those of LCFAs. FAs with a chain length of ≥ 20 are called very long-chain FAs (VLCFAs). VLCFAs are characteristically divided into saturated, monounsaturated, n-6 polyunsaturated, and n-3 polyunsaturated FAs (PUFAs), each of which exhibit distinct functions and properties. Most saturated and monounsaturated VLCFAs are sphingolipid components and so play important roles in skin barrier formation and neural functions (2, 3). PUFAs of the n-6 series (e.g., C20:4) are involved in the promotion of inflammation as precursors of eicosanoids (4). PUFAs of the n-3 series (e.g., C20:5 and C22:6) are effective in the prevention of arteriosclerosis, heart failure, and age-related macular degeneration by virtue of their roles in reducing plasma triglycerides, lowering blood pressure, and suppressing inflammation (5).

Sphingolipids are major lipid components of eukaryotic plasma membranes, along with glycerophospholipids and cholesterol. Ceramide (Cer), the sphingolipid backbone, comprises a long-chain base attached to FA via an amide bond. In mammalian tissues, C16:0 FA and C24 (C24:0 and C24:1) VLCFAs are the most common FAs. Sphingolipids characteristically carry a C24 FA. This long C24 moiety would be expected to interdigitate within the plasma membrane. Indeed, in one study, C24 lactosylceramide in the outer leaflet of the plasma membrane interacted

with the Src family kinase LYN, which is expressed and functions in leukocytes, anchored to the inner leaflet (6).

VLCFAs are produced from certain LCFAs, provided through diet or generated by FA synthase, or they are elongated from shorter VLCFAs by endoplasmic reticulum (ER) membrane-bound enzymes, which lengthen each chain with carbon units from FAs that have been converted to substrate-form acyl-CoAs (7). The FA elongase machinery comprises four distinct enzymes. The first type, known as elongases, condenses acyl-CoA and malonyl-CoA to produce 3-ketoacyl-CoA; this step is rate-limiting in VLCFA synthesis. To date, seven elongases (ELOVL1–7) have been identified in mammals and characterized (8, 9) (Table S1). The elongases ELOVL1–7 have each been shown to exhibit characteristic substrate specificities (8, 9) (Table S1), yet their precise roles in the VLCFA elongation pathways have not been completely determined. This dearth of information is due to insufficient or incomplete biochemical analyses, limited substrates, and inconsistent techniques among researchers (Table S1).

In addition to better understanding the roles of the ELOVLs and their substrate specificities in VLCFA elongation, it is necessary to also recognize the role of regulation in these pathways, as well as the interaction with other lipid systems. Cer synthases catalyze the synthesis of Cer from long-chain base and fatty acyl-CoA. Each of six mammalian Cer synthases (CERS1–6) exhibits its own characteristic substrate specificity toward certain fatty acyl-CoA(s) (2). Recent studies using *Cers2* knockout mice revealed that *Cers2* is the predominant Cer synthase for C24 Cer production (3, 10). These mice exhibited severe hepatopathy, myelin sheath defects, and cerebellar degeneration (3, 11).

In the presented studies, we have analyzed in vitro, detailed substrate specificities of all the ELOVLs and provide an overall summary of the VLCFA synthetic pathways. Especially significant is the demonstrated importance of ELOVL1 in the production of C24-CoA, the substrate of C24 sphingolipids. Of even more notable interest, we found a regulation of ELOVL1 by ceramide synthase CERS2 and established a close link between VLCFA elongation and C24 Cer production.

Results

Determination of Substrate Specificities of ELOVL Proteins. Although ELOVL1 has been predicted to be involved in the production of C24 sphingolipids based on studies using elongase-null yeast mutants expressing ELOVL1 (12), its precise substrate specificities toward acyl-CoAs have not been determined by direct, bio-

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chemical analysis. Moreover, the substrates for other ELOVLs have also not been precisely determined due to insufficient or incomplete biochemical analyses, limited substrates, and inconsistent techniques among researchers (Table S1). Therefore, we first determined the substrate specificity of each ELOVL protein under standardized conditions. For this purpose, we cloned the *ELOVL* genes and expressed them in HEK 293T cells as N-terminally triple FLAG (3xFLAG)-tagged proteins. By immunoblotting with an anti-FLAG antibody, ELOVL1, 2, 5, and 7 were each detected as a single band, however, two bands were observed for ELOVL3, 4, and 6 (Fig. S14). The upper bands represent their N-glycosylated forms, because these shifted to low molecular weight bands upon treatment with endoglycosidase H (Endo H) (Fig. S1B), which removes high mannose-type glycosylation. Indirect immunofluorescence microscopy revealed that all ELOVLs were localized in the ER (Fig. S2). This localization is reasonable, because VLCFA synthesis occurs there.

We performed in vitro elongase assays using total membrane fractions prepared from HEK 293T cells overproducing each ELOVL, and a mixture containing [¹⁴C]malonyl-CoA and one of 11 different acyl-CoAs [C16:0-, C18:0-, C18:1(n-9)-, C18:2(n-6)-, C18:3(n-3)-, C18:3(n-6)-, C20:0-, C20:4(n-6)-, C22:0-, C24:0-, or C26:0-CoA]. The ELOVL protein levels in the membrane fractions were determined to be expressed at similar levels by immunoblotting following treatment with Endo H (Fig. 1A). ELOVL1 elongated saturated C18:0-C26:0 acyl-CoAs, with the highest activity toward C22:0-CoA, yet had no elongation activity toward unsaturated acyl-CoA (Fig. 1B). Of the ELOVLs, ELOVL1 was the most potent elongase for C22:0-, C24:0-, and C26:0-CoAs. ELOVL3 and 7 also elongated C18:0-CoA, and generally exhibited similar substrate specificities (Fig. 1B). Their highest activities were toward C18-CoAs [especially C18:0-CoA for ELOVL3 and C18:3(n-3)-CoA for ELOVL7], relatively independently of the number of double bonds. Both also exhibited activities toward C16-C22 acyl-CoAs. ELOVL4 elongated C24:0- and C26:0-CoAs with weak but significant activities (Fig. 1B). The activity of ELOVL6 was extremely high toward C16:0-CoA (Fig. 1B). As reported previously (13–16), ELOVL2 and 5 acted specifically toward polyunsaturated acyl-CoAs and were the most potent elongases for C20:4(n-6)-CoA and C18:3(n-6)-CoAs, respectively (Fig. 1B).

In the above assays, the product FAs were separated by normal-phase TLC, which does not efficiently separate FAs with different chain lengths. Therefore, it remained unclear how many elongation cycles occurred in each reaction. For example, because ELOVL1 could elongate C18:0-C26:0 acyl-CoAs, we postulated that ELOVL1 elongated C18:0-CoA not only to C20:0-CoA but also further to form the longer acyl-CoAs. To test this possibility, we separated the product FAs of the elongase assays by reverse-phase TLC. We indeed found that substantial amounts of C24:0- and C26:0-CoA were produced by ELOVL1 when C18:0-CoA was used as a substrate (Fig. 1C). In contrast, ELOVL3 and 7 elongated C18:0-CoA mainly to C20:0-CoA, although low levels of C22:0-CoA were observed (Fig. 1C). Similarly, ELOVL1 but not ELOVL3 elongated C20:0-CoA efficiently to C24:0-CoA (Fig. 1C).

In the yeast *Saccharomyces cerevisiae*, VLCFAs are almost exclusively C26:0 FAs with or without α -hydroxylation, and no PUFAs exist. Yeast have three elongases, Elo1, Fen1 (Elo2), and Sur4 (Elo3). Fen1 and Sur4 elongate long-chain acyl-CoAs to C22:0-CoA (weakly to C24:0-CoA) and C26:0-CoA, respectively (17, 18). Because VLCFA production is essential for yeast growth, double deletion of *FEN1* and *SUR4* genes is lethal (19, 20). Reportedly, *ELOVL1* can rescue the lethality of Δ *fen1* Δ *sur4* cells (12). We confirmed this result, and additionally found that *ELOVL3* and *ELOVL7* can restore the growth defects of conditional Δ *fen1* Δ *sur4* cells, although weakly, especially for *ELOVL3* (Fig. S34); findings are detailed in the *SI Text*. Using

GC/MS, we further confirmed the production of C24:0-CoAs in cells expressing ELOVL1 (Fig. S3C). In summary, ELOVL1, 3, and 7 exhibit activities for elongating saturated long-chain acyl-CoAs to very long-chain acyl-CoAs.

Tissue-Specific Distribution of ELOVLs. To investigate the tissue-specific expression pattern of each *ELOVL* mRNA, we performed RT-PCR analyses using human cDNAs of 16 different tissues (Fig. 2). *ELOVL1*, *ELOVL5*, and *ELOVL6* mRNAs were expressed ubiquitously. *ELOVL4* mRNA was expressed in many tissues, but its expression levels varied, being highest in thymus, followed by testis, small intestine, ovary, and prostate. Little or no *ELOVL4* was expressed in heart, lung, liver, or leukocytes. The *ELOVL7* mRNA was also expressed in most tissues tested except heart and skeletal muscle. Expression in pancreas, kidney, prostate, and colon was high, whereas that in lung, ovary, spleen, and thymus was low. Two bands were produced by *ELOVL7*-specific primers. Sequencing analyses revealed that the upper band was a splicing isoform and contained an additional 119 bp nucleotides between 65G and 66A of the *ELOVL7* coding region. This sequence contained a stop codon, resulting in the production of a protein with very low molecular mass (3 kDa). The role of this splicing isoform is unclear, but it may be functionless. *ELOVL2* and *ELOVL3* mRNA expression was highly tissue specific. High expression of *ELOVL2* was observed only in liver and testis, with weak expression in pancreas, placenta, and prostate. Significant expression of *ELOVL3* was detected only in testis. These results indicate that each ELOVL exhibits a characteristic tissue-expression pattern, although expression levels of certain ELOVLs may be changed in certain tissues under different conditions.

ELOVL1 is Important for C24 Cer Synthesis. The in vitro results (Fig. 1B and C) and yeast complementation analyses (Fig. S3) (12) suggest that ELOVL1 is important for C24 sphingolipid synthesis, yet this possibility has not been proven using mammalian cells. To confirm this possibility, we performed knockdown analysis using HeLa cells and siRNA specific to *ELOVL1*. Total membrane fractions prepared from HeLa cells treated with *ELOVL1* siRNA exhibited significantly lower activities toward C18:0-, C20:0-, and C22:0-CoAs, but not toward C16:0- or C18:3(n-3)-CoA compared to control cells (Fig. 3A), agreeing with the in vitro results (Fig. 1B). Efficiency of the knockdown of mRNA levels by the *ELOVL1* siRNA was confirmed by RT-PCR analysis (Fig. 3B). In HeLa cells, neither *ELOVL2* nor *ELOVL4* is expressed, and the mRNA levels of the other expressed *ELOVLs* were not affected by the *ELOVL1* siRNA (Fig. S4). We also confirmed that in the human *ELOVL1* siRNA-treated HeLa cells, expression of mouse Elovl1 reversed the inhibitory effects on the elongase activities toward C20:0- and C22:0-CoAs (Fig. S5).

To examine the effect of the *ELOVL1* siRNA on the chain length of Cer, we next performed [³H]sphingosine labeling experiments. HeLa cells treated with control siRNA or *ELOVL1* siRNA were labeled with [³H]sphingosine, then lipids were extracted and separated by reverse-phase TLC. Control cells produced high levels of C24:1 Cer and, to a lesser extent, C24:0 Cer, whereas treatment with the *ELOVL1* siRNA resulted in a reduction in these C24 Cers and an increase in C16:0 and C18:0 Cers (Fig. 3C). Liquid chromatography (LC)/MS analysis measuring sphingomyelins (SMs) revealed that C24 SMs were abundant in the HeLa cells treated with the control siRNA, but were reduced in those treated with the *ELOVL1* siRNA; instead, C16:0 and C18:0 SMs were increased (Fig. 3D).

The above analyses indicate that ELOVL1 is important not only for C24:0 production but also for production of C24:1 sphingolipids. However, in the above in vitro analysis (Fig. 1B), we were not able to test the ELOVL1 activity toward C20:1(n-9)- or C22:1(n-9)-CoAs due to the unavailability of these acyl-CoAs. Therefore, we performed an in vitro elongase assay

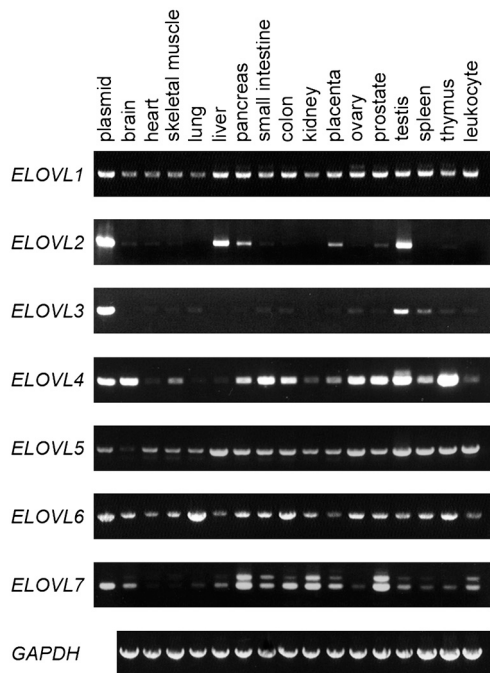


Fig. 2. Tissue-specific expression patterns of human *ELOVL* mRNAs. *ELOVL* and *GAPDH* cDNAs were amplified by PCR from human tissue cDNAs or from the pCE-puro 3xFLAG-*ELOVLx* plasmid using specific primers. Amplified fragments were separated by agarose gel electrophoresis and stained with ethidium bromide.

tant in the synthesis of both saturated and monounsaturated C24 sphingolipids. Based on our results (Fig. 1*B* and Fig. S6) and others' published reports (Table S1), we propose a model for mammalian VLCFA elongation pathways (Fig. 1*D*). However, precise determination of each pathway will require future biochemical and *in vivo* studies.

CERS2 Regulates C24-CoA Synthesis. Because C24-CoA is almost exclusively used for sphingolipid synthesis, we speculated that some link between sphingolipid synthesis and VLCFA elongation might exist. Therefore, we performed an *in vitro* VLCFA elongase assay in the presence of fumonisin B₁ (FB₁), a specific inhibitor of Cer synthesis. When C20:0- or C22:0-CoA was used as a substrate, FB₁ inhibited the elongation reaction (Fig. 4*A*). However, FB₁ had no effect on the elongation of C16:0-, C18:0-, or C18:3(n-3)-CoA.

In HeLa cells, CERS2, 4, and 5 are endogenously expressed (21), so we next determined which CERS was responsible for the FB₁-dependent inhibition of elongation, using knockdown analysis with siRNAs specific for *CERS2*, 4, or 5 (Fig. 4*B*). Neither *CERS4* nor *CERS5* siRNA affected the elongation of any acyl-CoAs tested. However, *CERS2* siRNA specifically inhibited the elongation of C20:0- and C22:0-CoAs (Fig. 4*C*); this effect was reversed by the introduction of mouse *Cers2* plasmid into the siRNA-treated HeLa cells (Fig. S7). *CERS2* is known to be important for C24 Cer synthesis (3, 22). Both of the reactions inhibited by FB₁ or *CERS2* siRNA, i.e., the elongations of C20:0- and C22:0-CoAs, are essential for the production of C24-CoA, the *CERS2* substrate. Remarkably, *ELOVL1* is responsible for both reactions (Fig. 1*B*). Therefore, we examined the interaction between *ELOVL1* and *CERS2*. FLAG-*ELOVL1* and HA-*CERS2* were expressed in HEK 293T cells, and cell extracts were subjected to coimmunoprecipitation with anti-FLAG antibodies. Two bands were detected by immunoblot for human *CERS2*, as previously observed for mouse *CerS2* (22), in which the upper band was determined to represent an *N*-glycosylation form. FLAG-*ELOVL1* specifically precipitated *CERS2* of an unglyco-

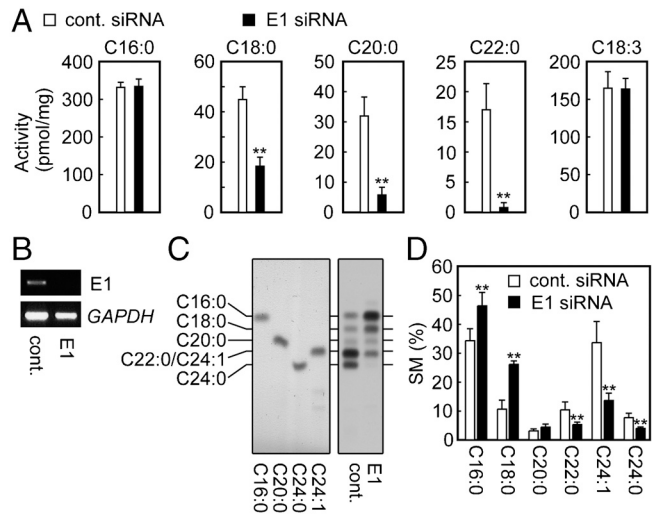


Fig. 3. *ELOVL1* is important for C24 sphingolipid production. HeLa cells were transfected with 16 nM control or *ELOVL1* siRNA 4 d prior to experiments. (A) Total membrane proteins (40 μ g) were incubated with the indicated acyl-CoA (50 μ M) and 0.025 μ Ci [¹⁴C]malonyl-CoA for 1 h at 37 $^{\circ}$ C. After termination of the reactions, lipids were saponified, acidified, extracted, and separated by TLC, followed by detection and quantification by a bioimaging analyzer BAS-2500. Values represent the mean \pm SD from three independent experiments. Statistically significant differences are indicated (***p* < 0.01; *t* test). (B) Total RNA was prepared from the transfected cells and subjected to RT-PCR using primers specific for *ELOVL1* or *GAPDH*. (C) Cells were labeled with 2 μ Ci [³H]sphingosine for 2 h at 37 $^{\circ}$ C. Lipids were extracted, separated by reverse-phase TLC, and detected by autoradiography (Right). The left panel shows C16:0 Cer, C20:0 Cer, C24:0 Cer, and C24:1 Cer standards, each at 10 nmol, separated by reverse-phase TLC, and stained with cupric acetate/phosphoric acid solution. (D) Lipids were extracted from the transfected cells and subjected to LC/ESI-MS/MS analysis. SM ions, determined as those losing the 60 Da neutral ion, were quantified. Values represent the relative amounts of certain SM species compared to total SM levels, and are the mean \pm SD from three independent experiments. Note that ionization efficiencies varied among molecular species of SMs. Statistically significant differences are indicated (***p* < 0.01; *t* test). E1, *ELOVL1*; cont., control.

sylated form (Fig. 4*D*). It is possible that glycosylation of *CERS2* regulates the interaction with *ELOVL1*. *CERS4*, which exhibits a substrate specificity similar to that of *CERS2*, did not interact with *ELOVL1* (Fig. 4*D*).

In yeast, enzymes involved in the VLCFA elongation cycle form an elongase complex (18). Similarly, in lysates of mammalian cells, we observed the coimmunoprecipitation of *ELOVL1* with HACD proteins, the enzymes responsible for the third reactions in the VLCFA elongation cycle (23). To investigate whether *ELOVL1* also interacts with other components of the VLCFA elongation machinery other than HACD proteins, we performed coimmunoprecipitation experiments using HA-tagged *ELOVL1* and FLAG-tagged 3-ketoacyl-CoA reductase (KAR) and *trans*-2,3-enoyl-CoA reductase (TER), the enzymes catalyzing the second and fourth reactions in the VLCFA elongation cycle, respectively. We found that both FLAG-KAR and FLAG-TER were coimmunoprecipitated with HA-*ELOVL1* (Fig. S8*A*), suggesting that mammals also form VLCFA elongase complex(es).

We next examined whether *CERS2* forms a complex with KAR, TER, or other *ELOVLs* by coimmunoprecipitation experiments using HA-tagged *CERS2* and FLAG-tagged KAR, TER, or *ELOVLs*. We found that HA-*CERS2* precipitated with both KAR and TER, as well as with each of the seven *ELOVLs* (Fig. S8*B*). This result suggests that *CERS2* may interact with *ELOVL1* not directly but indirectly via common factor(s) in the elongase complex(es), such as KAR, TER, or unidentified subunits.

C24 Sphingolipids Are Important for Membrane Microdomain Functions. Sphingolipids and cholesterol form membrane micro-

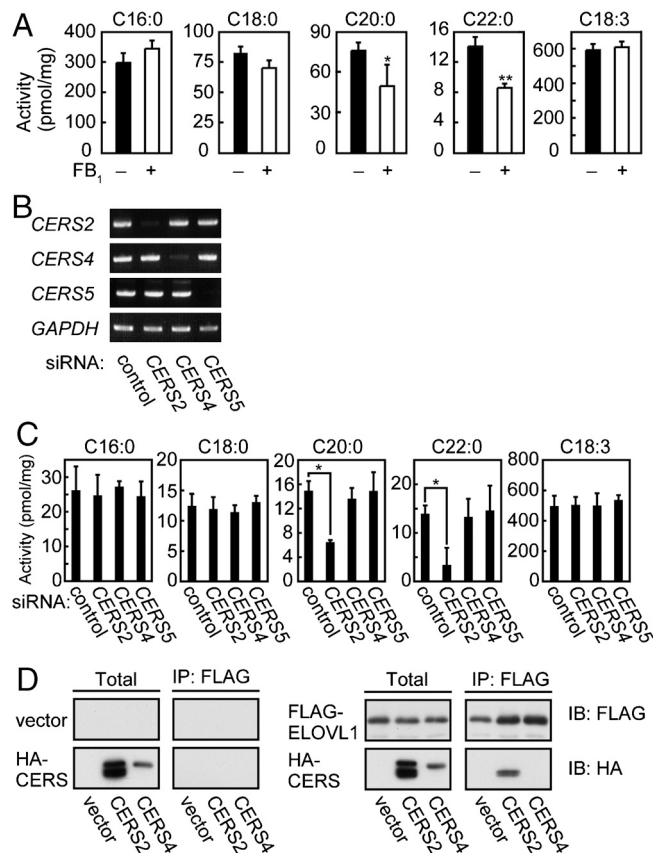


Fig. 4. CERS2 regulates ELOVL1 activity. (A) Total membrane proteins (40 μ g) prepared from HEK 293T cells were incubated with the indicated acyl-CoA (40 μ M) and 0.025 μ Ci [14 C]malonyl-CoA in the presence or absence of 20 μ M FB₁ for 1 h at 37 °C. After termination of the reactions, lipids were saponified, acidified, extracted, and separated by TLC, followed by detection and quantification by a bioimaging analyzer BAS-2500. Values represent the mean \pm SD from three independent experiments. Statistically significant differences are indicated (* p < 0.05, ** p < 0.01; t test). (B) HeLa cells were transfected with the indicated siRNAs (6 nM) 4 d prior to experiments. Total RNA was prepared then subjected to RT-PCR using primers specific for *CERS2*, *CERS4*, *CERS5*, or *GAPDH*. (C) Total membrane proteins (40 μ g) prepared from HeLa cells transfected with the indicated siRNAs were subjected to an in vitro elongase assay as in A using the indicated acyl-CoA (50 μ M) and 0.04 μ Ci [14 C]malonyl-CoA. Values represent the mean \pm SD from three independent experiments. Statistically significant differences are indicated (* p < 0.05; t test). (D) HEK 293T cells were transfected with a pcDNA3 HA-1 control vector, or a pcDNA3 HA-CERS2 or pcDNA3 HA-CERS4 plasmid, and with a pCE-puro 3xFLAG-ELOVL1 plasmid or pCE-puro 3xFLAG-1 control vector. Total cell lysates were prepared from the transfected cells and solubilized with 1% Triton X-100. Total lysates (Left) or proteins immunoprecipitated with anti-FLAG M2 agarose (Right) were subjected to immunoblotting with anti-FLAG or anti-HA antibodies. IP, immunoprecipitation; IB, immunoblotting; Total, total cell lysates.

domains, and an importance of C24 sphingolipids in microdomain functions has been suggested. Iwabuchi et al. found that C24 lactosylceramides are involved in the activation of LYN associated with membrane microdomains, by comparing cells having a high versus a low C24 lactosylceramide content and by loading exogenous C24 lactosylceramides into the cells having low C24 lactosylceramide content (24). With those results in mind, we tested whether a reduction in endogenous C24 sphingolipids by *ELOVL1* siRNA would affect the activation of LYN. HeLa cells expressing LYN-3xFLAG were treated with *ELOVL1* siRNA, and phosphorylated LYN, i.e., its activated form, was examined. Phosphorylated LYN was reduced to 29% in the *ELOVL1* siRNA-treated cells, compared to controls (Fig. 5). A similar effect was observed using siRNA specific for *CERS2*

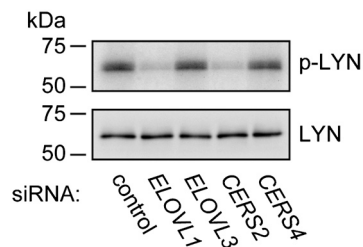


Fig. 5. Knockdown of *ELOVL1* mRNA causes a reduction in LYN activity. HeLa cells were transfected with control, *ELOVL1*, *ELOVL3*, *CERS2*, or *CERS4* siRNAs (15 nM) 4 d prior to experiments, and with the pCE-puro LYN-3xFLAG plasmid 2 d prior to experiments. Total lysates were prepared from the cells, solubilized with Triton X-100, and immunoprecipitated with anti-FLAG M2 antibodies. Immunoprecipitates were incubated with 5 μ Ci [γ - 32 P]ATP for 5 min at 37 °C, then separated by SDS-PAGE, and detected by autoradiography (Upper) or by immunoblotting with anti-FLAG antibodies (Lower). p-LYN, phosphorylated LYN.

(Fig. 5). Thus, the importance of C24 sphingolipids in the membrane microdomain functions was confirmed.

Discussion

Mammals have seven proteins, ELOVL1–7, that share sequence similarities to yeast VLCFA elongases. Synthesis of VLCFAs occurs by cycling through a four-step process, and the first, an elongation step, utilizes such elongases and is rate-limiting. There had been increasing evidence that each ELOVL exhibits characteristic substrate specificity (Table S1). However, an overall understanding of ELOVLs and the elongation pathway had not yet been recognized. In the study presented here, we performed comprehensive biochemical analyses using all ELOVLs and 11 different acyl-CoA substrates. Based on our results and others' published reports (Table S1), we herein update current and accepted knowledge regarding the FA elongation pathways in mammals (Fig. 1D).

In in vitro studies, ELOVL1 exhibited activities toward all of the saturated C18- to C26-CoAs as substrates, with the highest activity toward C22:0-CoA (Fig. 1B). Importantly, other ELOVLs, except ELOVL3, could not elongate C20:0- or C22:0-CoA. Because ELOVL3 is expressed only in limited tissues, such as testis, hair follicles, sebaceous glands, and brown adipose tissue in mice exposed to cold stress (Fig. 2) (25, 26), ELOVL1 may be the sole elongase responsible for the production of C22:0-CoA and C24:0-CoA in most tissues. In mammals, C24:0-CoA and C24:1-CoA are mainly used for C24 sphingolipid synthesis, and ELOVL1 has an important role in its synthesis, as determined here by knockdown analysis (Fig. 3 C and D). Sphingolipids with VLCFAs provide several important functions in mammalian physiology. For example, the C24 lactosylceramides found in microdomains are important for activation of LYN and cell signaling in neutrophils (24). It is notable, then that knockdown of the *ELOVL1* mRNA caused a reduction in the activity of LYN (Fig. 5). A recent report analyzing *Cers2* knockout mice revealed that a deficiency in C24 sphingolipid synthesis results in myelin sheath defects, cerebellar degeneration, and hepatopathy (3, 11). Moreover, the VLCFA moiety of sphingolipids may be important for determination in molecular species of sphingolipids, because C24 FAs are used in the gangliosides GM3 and GD3 but not in other gangliosides with more complex sugar structures (27). In further support of this notion, we recently revealed that VLCFA synthesis in yeast is important for the efficient production of complex sphingolipids (28).

Although membrane fractions overproducing ELOVL1 exhibited weak activity toward C18:0-CoA in vitro (Fig. 1B), knockdown of *ELOVL1* in HeLa cells resulted in greatly reduced (to ~40%) elongase activity toward C18:0-CoA (Fig. 3A) and in an accumulation of C18:0 SM (Fig. 3D). These results indicate that ELOVL1 is a major C18:0 elongase, at least in HeLa cells grown under our experimental conditions. It is possible that ELOVL1 is expressed more predominantly than other C18:0 elongases in HeLa cells.

Alternatively, perhaps overproduced ELOVL1 could not exert full activity in our assay conditions due to insufficient amounts of its regulatory components such as CERS2. We determined that elongation of C20:0- and C22:0-CoAs is regulated by CERS2 (Fig. 4C). Both elongations are essential steps for C24 Cer synthesis and are catalyzed by ELOVL1. Although the molecular mechanism of the regulation of ELOVL1 by CERS2 remains unclear, we speculate that CERS2 facilitates the release of the product generated by the ELOVL1 elongase complex, C24-CoA. Without the aid of CERS2, C24-CoA may be stuck within the elongase complex, leading to an inhibition of the next reaction round. Association of CERS2 with the elongase complex (Fig. 4D and Fig. S8B) may enable CERS2 to efficiently transfer C24-CoAs from the elongase complex to the catalytic site of CERS2, where a C24-CoA and a long-chain base react to produce C24 Cer. We hypothesize that this mechanism may ensure that the production of C24-CoA by elongation is coordinated with its utilization. Consistent with our results, a recent report demonstrated that the levels of C24-CoAs were nearly unchanged in *Cers2* knockout mice, whereas the other *Cers2* substrate dihydrosphingosine accumulated (10).

Our results update and enhance the overall picture of the VLCFA elongation cycles. Specific inhibitors for certain ELOVLs may be useful in studying or even treating several pathologies in which VLCFAs are involved; knowing the preferred substrates may aid the creation of such inhibitors. In an early example of such a feat, *Elovl6* knockout mice were found to exhibit marked protection from hyperinsulinemia, hyperglycemia, and hyperleptinemia (29). Based on this finding, a specific inhibitor for ELOVL6 was designed for utility as a pharmacological tool (30). Moreover, if certain drugs can induce a metabolic shift from LCFAs to VLCFAs, several metabolic disorder syndromes involving LCFAs may be improved. Future studies are required to elucidate the molecular mechanisms of the regulation and functions of specific ELOVL under certain pathologies.

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Materials and Methods

Detailed materials and methods used for all procedures are available in *SI Text*.

In Vitro FA Elongation Assays. In vitro FA elongation assays were performed essentially as described elsewhere (13) using total membrane fractions. Typical reaction mixtures of FA elongation assays contained total membrane fractions (20 µg protein), 50 µM acyl-CoA complexed with 0.2 mg/mL FA-free bovine serum albumin (Sigma), and 0.075 µCi [¹⁴C]malonyl-CoA (55 mCi/mmol; Moravek Biochemicals) in a 50 µL reaction mixture. When FAs were used in place of acyl-CoAs, reaction mixtures contained total membrane fractions (40 µg protein), 50 µM FAs, 200 µM CoA, 10 mM ATP, and 0.075 µCi [¹⁴C]malonyl-CoA.

Lipid Analysis by LC/MS. The amounts of SMs were determined by LC/electron spray ionization (ESI)-MS/MS essentially as described elsewhere (31, 32). Lipids were resolved by HPLC (Agilent 1100 series; Agilent Technologies) on a normal-phase column (Inertsil SIL100A 3 µm, 2.1 × 100 mm; GL Science). The ESI-MS/MS analyses were performed using an ion trap mass spectrometer LCQ Fleet (Thermo Fisher Scientific Inc). The data were analyzed and quantified using the XCalibur software (Thermo Fisher Scientific, Inc.).

RNA Interference. The control siRNA and siRNAs for *CERS2*, *CERS4*, and *CERS5* were purchased from Qiagen and have been described previously (21). The nucleotide sequences of *ELOVL1* siRNA were 5'-CCUGUACUACGGAUUUAUCUGC-3' (sense) and 5'-AGAUAAUCCGUAGUACAGGUA-3' (antisense), and the corresponding duplex siRNA was purchased from Sigma.

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