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Identification of acyl-CoA synthetases involved in the mammalian sphingosine 1-phosphate metabolic pathway

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Highlights

- Eight ACSs exhibited activities toward the S1P metabolite *trans*-2-hecadecenoic acid.
- ACS isozymes involved in S1P metabolism were localized in the ER.
- Inhibition of ACSs caused accumulation of *trans*-2-hexadecenoic acid.

ABSTRACT

Sphingosine 1-phosphate (S1P) plays important roles both as a bioactive lipid molecule and an intermediate of the sphingolipid-to-glycerophospholipid metabolic pathway. To identify human acyl-CoA synthetases (ACSs) involved in S1P metabolism, we cloned all 26 human ACS genes and examined their abilities to restore deficient sphingolipid-to-glycerophospholipid metabolism in a yeast mutant lacking two ACS genes, *FAA1* and *FAA4*. Here, in addition to the previously identified ACSL family members (ACSL1, 3, 4, 5, and 6), we found that ACSVL1, ACSVL4, and ACSBG1 also restored metabolism. All 8 ACSs were localized either exclusively or partly to the endoplasmic reticulum (ER), where S1P metabolism takes place. We previously proposed the entire S1P metabolic pathway from results obtained using yeast cells, i.e., S1P is metabolized to glycerophospholipids via *trans*-2-hexadecenal, *trans*-2-hexadecenoic acid, *trans*-2-hexadecenoyl-CoA, and palmitoyl-CoA. However, as S1P is not a naturally occurring long-chain base 1-phosphate in yeast, the validity of this pathway required further verification using mammalian cells. In the present study, we treated HeLa cells with the ACS inhibitor triacsin C and found that inhibition of ACSs resulted in accumulation of *trans*-2-hexadecenoic acid as in ACS mutant yeast. From these results, we conclude that S1P is metabolized by a common pathway in eukaryotes.

Keywords: acyl-CoA synthetase, glycerophospholipid, sphingolipid, sphingosine 1 - phosphate

Abbreviations

ACS, acyl-CoA synthetase; ACSBG, ACS bubblegum; ACSL, ACS long-chain; ACSM, ACS medium-chain; ACSS, ACS short-chain; ACSVL, ACS very long-chain; DHS, dihydrosphingosine; DHS1P, dihydrosphingosine 1-phosphate; ER, endoplasmic reticulum; FA, fatty acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPC, inositol phosphorylceramide; LCB, long-chain base; LCBP, LCB 1-phosphate; LCFA, long-chain fatty acid; MIPC, mannosylinositol phosphorylceramide; M(IP)₂C, mannosyldiinositol phosphorylceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SC, synthetic complete; SPH, sphingosine; S1P, sphingosine 1-phosphate; VLCFA, very-long chain fatty acid.

1. Introduction

Sphingolipids are one of the major lipid components of eukaryotic plasma membranes. The hydrophobic backbone of sphingolipid ceramide is composed of a long-chain base (LCB) and a fatty acid (FA). Sphingosine (SPH) is the major LCB in mammals and its phosphorylated product sphingosine 1-phosphate (S1P) functions both as a bioactive lipid molecule and as a metabolic intermediate of the conversion of sphingolipids to glycerophospholipids [1]. As a bioactive lipid molecule, extracellular S1P induces several cellular responses, such as cell migration, proliferation, adherens junction assembly, and cytoskeletal remodeling, through binding to one of the G-protein coupled S1P receptors (S1P₁-S1P₅) [2]. This action of S1P is important for the egress of T lymphocytes from the thymus and secondary lymphoid tissues and has already been utilized clinically as a therapeutic agent (Fingolimod) for multiple sclerosis [3].

S1P plays important roles as a key metabolic intermediate in the sphingolipid-to-glycerophospholipid metabolic pathway [1]. Since the S1P metabolic pathway is the sole pathway allowing conversion of the LCB portion of sphingolipids to acyl-CoAs and further to glycerophospholipids, its failure to function may lead to aberrant sphingolipid homeostasis. Indeed, knockout mice for the *Spl* (*Sgpl1*) gene, which encodes S1P lyase that catalyzes the first, irreversible step of the S1P metabolic pathway, have a pleiotropic phenotype, including abnormal lipid homeostasis in the liver, brain, and adipose tissue, myeloid cell hyperplasia, skeletal and hematological dysfunctions, and lesions in the lung, heart, urinary tract, and bone, and these mice die approximately one month after birth [4,5].

In a previous study, we proposed the entire S1P metabolic pathway as follows [6]. Following cleavage of S1P to a fatty aldehyde *trans*-2-hexadecenal and phosphoethanolamine by S1P lyase, the resulting *trans*-2-hexadecenal is oxidized to *trans*-2-hexadecenoic acid by the fatty aldehyde dehydrogenase ALDH3A2. *Trans*-2-hexadecenoic acid is then converted to *trans*-2-hexadecenoyl-CoA by acyl-CoA synthetases (ACSSs), followed by reduction to palmitoyl-CoA by an unidentified

trans-2-enoyl-CoA reductase. Finally, palmitoyl-CoA is converted to several lipids, mainly glycerophospholipids.

ACSSs catalyze the conversion of FAs to their active form acyl-CoAs. The human genome codes for 26 ACS isozymes, which are classified into six subfamilies based on their substrate specificities toward the chain length of FAs and on sequence similarity [7] (Fig. 1A): the ACS short-chain (ACSS) subfamily (ACSS1, ACSS2, and ACSS3; substrates, C2 to C4 FAs), ACS medium-chain (ACSM) subfamily (ACSM1, ACSM2A, ACSM2B, ACSM3-5; substrates, C6 to C10 FAs), ACS long-chain (ACSL) subfamily (ACSL1 and ACSL3-6; substrates, C12 to C18 FAs), ACS very long-chain (ACSVL) subfamily (ACSVL1-6; substrates, C20 to C26 FAs), ACS bubblegum (ACSBG) subfamily (ACSBG1 and ACSBG2), and ACSF subfamily (ACSF1-4). ACSBG1 is active toward LCFAs and VLCFAs [8,9], and ACSBG2 exhibits activities toward C18:1 and C18:2 FAs, but not toward C16:0 [10]. The substrate of ACSF2 is C8:0 FA, whereas those of ACSF3 are malonate and methylmalonate [7]. The substrates of ACSF1 and ACSF4 have not been identified.

The LCBs of *Saccharomyces cerevisiae* yeast are dihydrosphingosine (DHS) and phytosphingosine (4-hydroxydihydrosphingosine) [11]. These LCBs are used for sphingolipid synthesis or converted to LCB 1-phosphates (LCBPs) by LCB kinases. DHS is also metabolized to glycerophospholipids after conversion to dihydrosphingosine 1-phosphate (DHS1P) by essentially the same pathway as the S1P metabolic pathway [6], whereas the details of phytosphingosine metabolism remain unclear. Although yeast do not produce SPH endogenously, exogenously added SPH is imported into cells and converted to glycerophospholipid after conversion to S1P as in mammals [6]. We previously demonstrated that SPH was not metabolized to glycerophospholipids in a yeast mutant containing deletion of two ACS genes, *FAA1* and *FAA4* ($\Delta faa1 \Delta faa4$ cells), among 7 yeast ACS genes [6]. Expression of human ACSL family members (*ACSL1*, *ACSL3*, *ACSL4*, *ACSL5*, or *ACSL6*) in $\Delta faa1 \Delta faa4$ cells restored the deficient SPH-to-glycerophospholipid conversion [6], suggesting involvement of ACSL family members in S1P metabolism.

However, in that study, the role of other human ACSs was not examined.

In $\Delta faa1 \Delta faa4$ cells, *trans*-2-hexadecenoic acid accumulated as an intermediate of S1P metabolism, suggesting that S1P metabolism includes conversion of *trans*-2-hexadecenoic acid to *trans*-2-hexadecenoyl-CoA by ACSs [6]. Although we suspect that the same S1P metabolic pathway also occurs in mammals, this must be verified using mammalian cells. In the present study, we revealed that ACSVL1, ACSVL4, and ACSBG1 were also involved in S1P metabolism in addition to previously identified ACSL family members, indicating that multiple ACSs contribute to S1P metabolism in mammals. Furthermore, treatment with the ACS inhibitor triacsin C in HeLa cells resulted in accumulation of *trans*-2-hexadecenoic acid. Therefore, these results demonstrated that the S1P metabolic pathway is conserved in yeast and mammals.

2. Materials and methods

2.1. Yeast strain and media

The *Saccharomyces cerevisiae* yeast strain AOY13 ($\Delta faa1 \Delta faa4$) was described previously [6]. Cells were grown in synthetic complete (SC) medium lacking uracil (0.67% yeast nitrogen base, 2% D-glucose, 0.5 % casamino acids, 20 mg/l adenine, and 20 mg/l tryptophan).

2.2. Cell culture, transfection, and plasmids

HeLa cells were grown in Dulbecco's modified Eagle's medium (D6049; Sigma, St. Louis, MO) containing 10% FCS and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). Transfections were conducted using Lipofectamine Plus Reagent (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Construction of plasmids is described in the Supplementary Information.

2.3. [^3H]LCB labeling assays

Yeast cells and HeLa cells were labeled with [4,5- ^3H] DHS (American Radiolabeled Chemicals, St. Louis, MO) or [11,12- ^3H]SPH, as described previously [6]. [11,12- ^3H]SPH was prepared from [9,10- ^3H]palmitic acid (American Radiolabeled Chemicals), as described previously [6].

2.4. Immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed as described previously [12]. For Nile red staining, cells were incubated with 1 µg/ml Nile red (Wako Pure Chemical Industries) for 30 min at room temperature, followed by blocking with BSA and staining with antibodies. Anti-calreticulin (1/400 dilution; Enzo Life Sciences, Farmingdale, NY) and anti-FLAG M2 (0.5 µg/ml) antibodies were used as primary antibodies. Alexa Fluor 488-conjugated anti-rabbit IgG (H+L) antibodies and Alexa Fluor 594-conjugated anti-mouse IgG (H+L) antibodies (each used at 5 µg/ml; Life Technologies) were used as

secondary antibodies. Fluorescence images were obtained with a Leica DM5000B microscope (Leica Microsystems, Wetzlar, Germany).

3. Results

3.1. ACSVL1, ACSVL4, and ACSBG1, in addition to ACSL members, restore SPH/S1P metabolism in ACS mutant yeast

We previously revealed that ectopic expression of the human ACSL family proteins ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6 in $\Delta faa1 \Delta faa4$ yeast cells restored deficient LCB-to-glycerophospholipid metabolism [6]. However, in that study, the potential involvement of other ACS family members (ACSS, ACSM, ACSVL, ACSBG, and ACSF family) was not examined. Therefore, we expanded analyses to include all human ACS isozymes in the present study. Each of the 26 human ACS genes was cloned into the yeast expression vector, producing the N-terminal 3xFLAG-tagged protein, which was then introduced into the $\Delta faa1 \Delta faa4$ cells. We confirmed that expression of all ACSs was present by immunoblotting with an anti-FLAG antibody, although expression of ACSL3 was extremely low (Fig. 1B). Using these cells, we performed [4,5-³H]DHS labeling experiments. DHS is a natural LCB in yeast and is metabolized in essentially the same way as SPH [6]. DHS is phosphorylated to DHS1P, cleaved to hexadecanal, oxidized to palmitic acid, and activated to palmitoyl-CoA by ACSs, before it is converted to glycerophospholipids [6]. In wild type cells, DHS was metabolized both to sphingolipids (including ceramide, inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC), and mannosyldiinositol phosphorylceramide (M(IP)₂C)) and glycerophospholipids (including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS)), whereas DHS was metabolized only to sphingolipids in $\Delta faa1 \Delta faa4$ cells (Fig. 1C–E, vector). As reported previously [6], expression of either of the ACSL members restored DHS-to-glycerophospholipid metabolism (Fig. 1C), although complementation by ACSL3 was weak due to its low expression. Both ACSL4 and ACSL5 have two splicing variants (variant 1 (v1) and variant 2 (v2)). Our previous report tested only the v1 isoforms for each of these proteins. Here, we demonstrated that both v1 and v2 isoforms were active in DHS/DHS1P metabolism (Fig. 1C). Some ACSBG and ACSVL members also restored deficient DHS metabolism.

ACSBG1 and ACSVL4 expression almost completely recovered the metabolism to the same level as ACSL members (Fig. 1D and E). ACSVL1 exhibited weak activity (Fig. 1E), whereas little or no restoration activities were observed for other ACSBG and ACSVL members. No ACSS, ACSM, or ACSF members were found to restore DHS/DHS1P metabolism (Fig. 1D and E).

In the SPH/S1P metabolic pathway, *trans*-2-hexadecenoic acid is converted to *trans*-2-hexadecenoyl-CoA by ACSs. Although a number of FAs have been used as substrates for *in vitro* ACS assays conducted for several ACSs, such assays have not been performed using *trans*-2-hexadecenoic acid. To examine which ACSs were responsible for the conversion of *trans*-2-hexadecenoic acid to *trans*-2-hexadecenoyl-CoA in the SPH/S1P metabolic pathway, we next subjected the ACSs that demonstrated activities in DHS/DHS1P metabolism (ACSL1, ACSL3, ACSL4, ACSL5, ACSL6, ACSVL1, ACSVL4, and ACSBG1) to [11,12-³H]SPH labeling experiments. As reported previously [6], $\Delta faa1 \Delta faa4$ cells bearing the vector alone could not convert [11,12-³H]SPH to glycerophospholipids, whereas introduction of either of the ACSL family members restored the metabolism (Fig. 2). Again, the restoration by ACSL3 was weak due to low expression. ACSBG1 and ACSVL4 also exhibited high restoration activities, while the activity of ACSVL1 was weak. These results suggest that ACSL1, ACSL3, ACSL4, ACSL5, ACSL6, ACSVL4, and ACSBG1 are the main ACS family members involved in the S1P metabolic pathway.

3.2. ACS isozymes involved in S1P metabolism are localized in the endoplasmic reticulum (ER)

S1P metabolism occurs in the ER, as the S1P lyase SPL, the fatty aldehyde dehydrogenase ALDH3A2, and several acyltransferases involved in the synthesis of glycerophospholipids are localized in the ER [1]. Therefore, ACSs responsible for S1P metabolism should also be localized in the ER for efficient metabolic flow. It has been reported that each ACS isozyme exhibits characteristic intracellular localization, such as in the ER, plasma membrane, mitochondria, and peroxisome [7,13,14]. However, the precise localization of most ACS

isozymes remains unclear since inconsistent localization patterns have been reported among researchers using different approaches. Therefore, we compared the localization of the ACSs using a uniform method. Indirect immunofluorescent microscopic analyses revealed that ACSL1, ACSL4 v2, ACSL6, ACSVL1, and ACSVL4 expression exhibited reticular structures that resembled the ER (Fig. 3A). Indeed, their expression was found to co-localize with the ER marker calreticulin. ACSL3 expression exhibited the reticular ER structure as well as some ring-like structures. Since ACSL3 is reportedly localized to intracellular lipid droplets [15], we visualized these lipid droplets using Nile red dye. We found that ACSL3 surrounded the Nile red-stained lipid droplets, while the negative control ACSL1 did not (Fig. 3B). Collectively, this indicates that ACSL3 is localized both to the ER and in lipid droplets. Although the ACSL4 v2 isoform was localized to the ER almost exclusively, the ACSL4 v1 isoform was mainly localized in the plasma membrane and partially in the ER. The difference between the v1 and v2 isoforms resides in their N-termini. The N-terminus of ACSL4 v1 is 41 amino acids shorter than that of v2 [7]. Although ACSL5 v1 and ACSL5 v2 were mainly localized to the ER, they were also found to be expressed in the plasma membrane. ACSBG1 was distributed both in the ER and the plasma membrane. In conclusion, all ACSs examined were either exclusively or partly localized to the ER. Among ACLs exhibiting high activities in the S1P metabolic pathway (Fig. 2), ACSL1, ACSL4 v2, ACSL6, and ACSVL4 were localized almost exclusively to the ER, suggesting that their contribution to S1P metabolism may be high.

Since S1P metabolism occurs ubiquitously [1], ACSs involved in S1P metabolism are expected to be expressed widely. Here, we investigated the tissue expression patterns of *ACSL1*, *ACSL3*, *ACSL4*, *ACSL5*, *ACSL6*, *ACSVL1*, *ACSVL4*, and *ACSBG1* mRNAs by RT-PCR (Supplementary Fig. S1). *ACSL1*, *ACSL3*, *ACSL4*, and *ACSVL4* mRNAs were detected in all tissues examined, indicating ubiquitous expression. *ACSL5* mRNA was expressed in most tissues examined, but not in the brain, kidney, or skeletal muscle. The expression of *ACSVL1* mRNA in the kidney and liver was high, whereas little expression was observed in the heart, leukocyte, ovary, skeletal muscle, or thymus. Expression of

ACSL6 and *ACSBG1* mRNAs were highly tissue-specific, with *ACSL6* mRNA highly expressed in the testis and weakly in the brain and prostate, while expression of *ACSBG1* mRNA was restricted to the brain and testis.

3.3. *Trans*-2-hexadecenoic acid is the intermediate of the S1P metabolic pathway in mammals

We previously detected *trans*-2-hexadecenoic acid as an intermediate of the S1P metabolic pathway in yeast ($\Delta faa1 \Delta faa4$ cells) [6], leading to proposal of the entire S1P metabolic pathway. However, detection of *trans*-2-hexadecenoic acid in mammalian cells under ACS-inhibited conditions was not tested in that study. In the present study, to detect the FA intermediate of S1P metabolism in mammals, we used the ACS inhibitor triacsin C [16]. HeLa cells were labeled with either [^3H]DHS or [^3H]SPH in the presence or absence of triacsin C. After labeling, lipids were extracted, treated with or without alkaline, and separated by normal-phase TLC. Alkaline treatment causes hydrolysis of the ester-linkage in glycerophospholipids and liberates FAs, whereas sphingolipids are resistant to alkaline treatment. Incubation with triacsin C reduced conversion of both DHS and SPH to glycerophospholipids (PC, PE, PI, and PS), whereas metabolism of sphingolipids (glucosylceramide, lactosylceramide, and sphingomyelin) was almost unaffected (Fig. 4A). Thus, inhibition by triacsin C was specific to LCB-to-glycerophospholipid conversion, eliminating the possibility that this inhibition was caused by cytotoxic effects of triacsin C.

We next subjected the labeled products to *trans*-methylation, by which free FAs and FAs in glycerophospholipids were converted to FA methylesters (FAMES). The chain-length of the FAMES was then determined by TLC separation using reverse-phase TLC (Fig. 4B). We found that DHS was mainly converted to C16:0 FA (palmitic acid/hexadecanoic acid; C16:0-methylester (ME) in Fig. 4B), while some was converted to *cis*-9-C16:1 FA (palmitoleic acid/*cis*-9-hexadecenoic acid; *cis*-C16:1-ME in Fig. 4B) via desaturation by $\Delta 9$ desaturase and to C18:0 FA (stearic acid/octadecanoic acid; C18:0-ME in Fig. 4B) via FA elongation. We also subjected half of the FAME products to double-bond cleavage assays

using KIO_4 and KMnO_4 , where carbon-carbon double bonds undergo oxidation to form two carboxylic acids [17]. As expected, saturated C16:0-ME and C18:0-ME were resistant to the $\text{KIO}_4/\text{KMnO}_4$ treatment, whereas double bond-containing *cis*-9-C16:1-ME was cleaved to nonanedioic acid ME (NA-ME in Fig. 4B) and unlabeled C7:0 FA (Fig. 4B and C).

SPH appeared to be similarly metabolized to FAs as DHS, based on the detection of C16:0-ME, *cis*-9-C16:1-ME, and C18:0-ME (Fig. 4B). Existence of *trans*-2-C16:1-ME was unclear due to its coincidence with C16:0-ME in reverse-phase TLC. Although the *cis*-9 double bond of *cis*-9-C16:1-ME caused a large bending in structure and different migration pattern compared to C16:0-ME in reverse phase TLC, the 2-*trans* double bond had little effect on structure or TLC mobility (Fig. 4B, middle and right panels). However, treatment with $\text{KIO}_4/\text{KMnO}_4$ could distinguish between C16:0-ME and *trans*-2-C16:1-ME. The C16:0-ME standard was resistant to $\text{KIO}_4/\text{KMnO}_4$, whereas the *trans*-2-C16:1-ME standard was converted to C14:0 FA (myristic acid) and unlabeled ethanedioic acid ME (Fig. 4B and C). When ME products of the [11,12- ^3H]SPH metabolite were treated with $\text{KIO}_4/\text{KMnO}_4$, C14:0 FA was indeed detected, indicating the existence of *trans*-2-hexadecenoic acid that was dependent on the triacsin C treatment (Fig. 4B). These results indicate that *trans*-2-hexadecenoic acid is indeed the metabolic intermediate of SPH/S1P in mammals as is observed in yeast.

4. Discussion

We previously reported that S1P is metabolized to glycerophospholipids in yeast through sequential enzyme reactions: cleavage by LCBP lyase, oxidation by fatty aldehyde dehydrogenase, activation by ACS, and reduction by *trans*-2-enoyl-CoA reductase [6]. However, since SPH/S1P is not a naturally occurring LCB/LCBP in yeast, this proposed S1P metabolic pathway needs to be confirmed in mammalian cells. We previously revealed that ACSL family members are involved in the S1P metabolic pathway [6]. In the present study, we expanded our analysis to include all ACSs in mammals and found that ACSVL1, ACSVL4, and ACSBG1, in addition to ACSL family proteins, mediate LCBP metabolism (Figs. 1 and 2). This high redundancy of ACSs in LCBP metabolism made it difficult to achieve efficient knockdown of all involved ACSs simultaneously in mammalian cells by multiple siRNAs, leading to unsuccessful inhibition of LCBP metabolism (data not shown). However, we could inhibit LCBP metabolism using the ACS inhibitor triacsin C (Fig. 4A). Importantly, we detected *trans*-2-hexadecenoic acid as a metabolic intermediate under S1P metabolism-inhibited conditions (Fig. 4B), confirming the same S1P metabolic pathway exists as in yeast.

The LCBP metabolic pathway, as well as involved enzymes (LCB/SPH kinases, LCBP/S1P lyases, fatty aldehyde dehydrogenases, and ACSs), are conserved among eukaryotes. Although S1P also functions as a lipid mediator through specific receptors in mammals, such receptors do not exist in yeast. The S1P receptor first appears evolutionally in chordates, indicating a much older origin of LCBP function as metabolic intermediates than as lipid mediators [18]. Furthermore, S1P metabolism occurs throughout mammalian tissues [1]. In the present study, we revealed that the expression of *ACSL1*, *ACSL3*, *ACSL4*, and *ACSVL4* mRNAs were also expressed in a wide variety of tissues (Supplementary Fig. S1). The S1P metabolic pathway takes place in the ER, considering the localization of the S1P lyase SPL, the fatty aldehyde dehydrogenase ALDH3A2, and the acyltransferases involved in glycerophospholipid synthesis. Among the widely expressed ACSs, *ACSL1*, *ACSL4* v2, and *ACSVL4* were localized to the ER almost exclusively, whereas *ACSL3* and

ACSL5 exhibited only partial localization to the ER (Fig. 3). Thus, ACSL1, ACSL4 v2, and ACSVL4 may be the key ACSs involved in S1P metabolism in most tissues, although other ACSs may also contribute.

Triacsin C treatment caused inhibition of S1P metabolism and accumulation of *trans*-2-hexadecenoic acid in HeLa cells (Fig. 4A and B). Among ACS isozymes which restored LCBP metabolism in $\Delta faa1 \Delta faa4$ cells, *ACSL1*, *ACSL3*, *ACSL4*, and *ACSVL1* mRNAs were expressed at high or intermediate levels in HeLa cells, while the expressions of *ACSL5*, *ACSBG1*, and *ACSVL4* mRNA were low (Supplementary Fig. S2). We could not detect *ACSL6* mRNA expression. Triacsin C has been reported to inhibit ACSL1, ACSL3, ACSL4, ACSL5, and ACSVL4 [19], whereas its effect on ACSVL1 or ACSBG1 has not been tested. Triacsin C treatment did not completely block LCBP metabolism in HeLa cells and the levels of accumulated *trans*-2-hexadecenal were relatively low (Fig. 4B). We speculate that the partial blockage of LCBP metabolism may be due to incomplete inhibition of ACSs by triacsin C at a sub-optimal concentration, as well as the relatively short incubation time (1 h), which was implemented to avoid indirect effects on cells such as growth defects and changes in global lipid metabolism.

Although existence of the S1P metabolic pathway was first reported in the late 1960s [20], the precise reactions of this pathway and the involved genes remained unresolved until our recent findings using yeast cells [6]. Our present studies have enormous significance in confirming that this elucidated pathway is indeed conserved in mammals. Further studies are required to identify the *trans*-2-enoyl-CoA reductase that catalyzes the last step of the S1P metabolic pathway and to reveal the regulatory mechanisms of this pathway.

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Figure legends

Fig. 1. ACSL family members, ACSVL4, and ACSBG1 restore DHS-to-glycerophospholipid metabolism in $\Delta faa1 \Delta faa4$ cells. (A) The phylogenetic tree of human ACSs was illustrated using ClustalW software (<http://www.genome.jp/tools/clustalw/>). (B–D) AOY13 ($\Delta faa1 \Delta faa4$) cells harboring a plasmid encoding the 3xFLAG-tagged ACS gene as indicated were grown in SC medium lacking uracil at 30 °C. (B) Cell lysates were prepared and subjected to immunoblotting with an anti-FLAG antibody or anti-Pgk1 antibody, which was used as a protein loading control. (C–D) Cells were labeled with 0.04 μ Ci [4,5- 3 H]DHS for 2 h at 30 °C. Lipids were extracted and separated by TLC with chloroform/methanol/4.2 N ammonia (9:7:2, v/v). vec, vector; L, ACSL; S, ACSS; M, ACSM; BG, ACSBG; VL, ACSVL; F, ACSF.

Fig. 2. ACSL family members, ACSVL4, and ACSBG1 complement the deficient SPH-to-glycerophospholipid metabolism in $\Delta faa1 \Delta faa4$ cells. AOY13 ($\Delta faa1 \Delta faa4$) mutant cells harboring a plasmid encoding the 3xFLAG-tagged ACS gene as indicated were grown in SC medium lacking uracil, and labeled with 0.04 μ Ci [11,12- 3 H]SPH for 2 h at 30 °C. Lipids were extracted and separated by TLC with chloroform/methanol/4.2 N ammonia (9:7:2, v/v).

Fig. 3. ACS isozymes involved in LCB-to-glycerophospholipid metabolism are localized to the ER. HeLa cells were transfected with a plasmid encoding the 3xFLAG-tagged ACS gene as indicated. Forty-eight hours after transfection, cells were fixed with formaldehyde and permeabilized with 0.1% Triton X-100. (A) Cells were stained with anti-FLAG antibody, anti-calreticulin antibody, and the DNA-staining reagent DAPI. The left panels depict cells stained with anti-FLAG antibody (red), the middle panels show cells stained with anti-calreticulin antibody (red) and the right panels are the merged images of anti-FLAG- and anti-calreticulin-stained cell that have also been stained with the DNA-staining reagent DAPI (blue) (B) Cells were stained with Nile red (red, middle panels) and anti-FLAG

antibody (green, left panels). The right panel shows the merge of the left and middle panels. Fluorescence images were obtained using a Leica DM5000B microscope. Calibration bar, 10 μ m.

Fig. 4. *Trans*-2-hexadecenoic acid is the intermediate of the S1P metabolic pathway in mammals. (A and B) HeLa cells were labeled with 0.1 μ Ci [4,5- 3 H]DHS or [11,12- 3 H]SPH for 4 h at 37 $^{\circ}$ C. Triacsin C (20 μ M) was added to cells 1 h before the labeling. (A) Lipids were extracted, treated with or without an alkaline solution, and separated by normal-phase TLC with 1-butanol/acetic acid/water (3:1:1, v/v). GlcCer, glucosylceramide; LacCer, lactosylceramide; SM, sphingomyelin. (B) Lipids were extracted and subjected to *trans*-methylation. The generated FAMES were isolated by hexane/methanol phase separation, treated with or without KMnO₄/KIO₄, and separated by reverse-phase TLC with chloroform/methanol/water (5:15:1, v/v). The standards *trans*-2-[9,10- 3 H]hexadecenic acid [6] and [9,10- 3 H]palmitic acid (American Radiolabeled Chemicals) were processed in the same way (middle and right panel). Asterisks indicate unidentified lipids, which may be derived from *trans*-2-hexadecenoic acid metabolites. (C) Reactions in (B) were illustrated for *trans*-2-hexadecenoic acid (*trans*-2-C16:1-FA) derived from [11,12- 3 H]SPH and *cis*-9-hexadecenoic acid (*cis*-9-C16:1-FA) derived from [11,12- 3 H]SPH and [4,5- 3 H]DHS. Closed and open circles indicate labeled hydrogens (tritiums) derived from [11,12- 3 H]SPH and [4,5- 3 H]DHS, respectively. Note that the tritiums in *cis*-9-hexadecenoic acid derived from [11,12- 3 H]SPH were removed during the methyl esterification. NA, nonanedioic acid; Me, methyl group.

A

Western blot analysis showing the expression of FLAG-tagged proteins (top panel) and Pgk1 (bottom panel) as a loading control. The top panel is probed with anti-FLAG antibody, showing bands for various proteins labeled L1 through F4. The bottom panel is probed with anti-Pgk1 antibody, showing a single band for Pgk1 across all lanes. Molecular weight markers are indicated on the left (kDa).

Thin layer chromatography (TLC) analysis of lipid composition in vesicles. The image shows a TLC plate with lanes labeled vec, L1, L3, L4 v1, L4 v2, L5 v1, L5 v2, and L6. The y-axis is labeled with lipid species: Cer, DHS, PE, FA, PC, PS/PI, IPC, MIPC, DHS1P, M(IP)₂C, and origin. The x-axis labels are at the bottom of the plate.

Figure 2

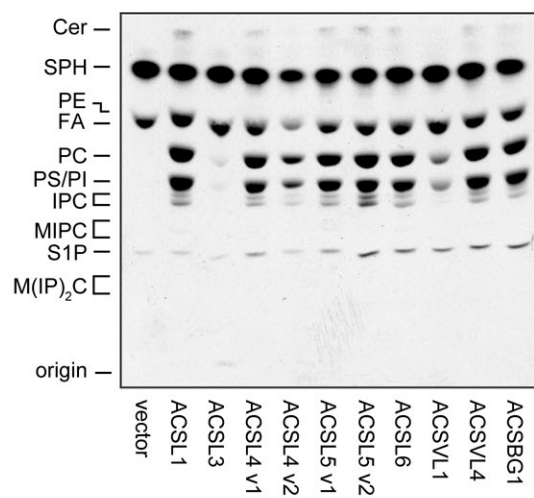


Figure 3

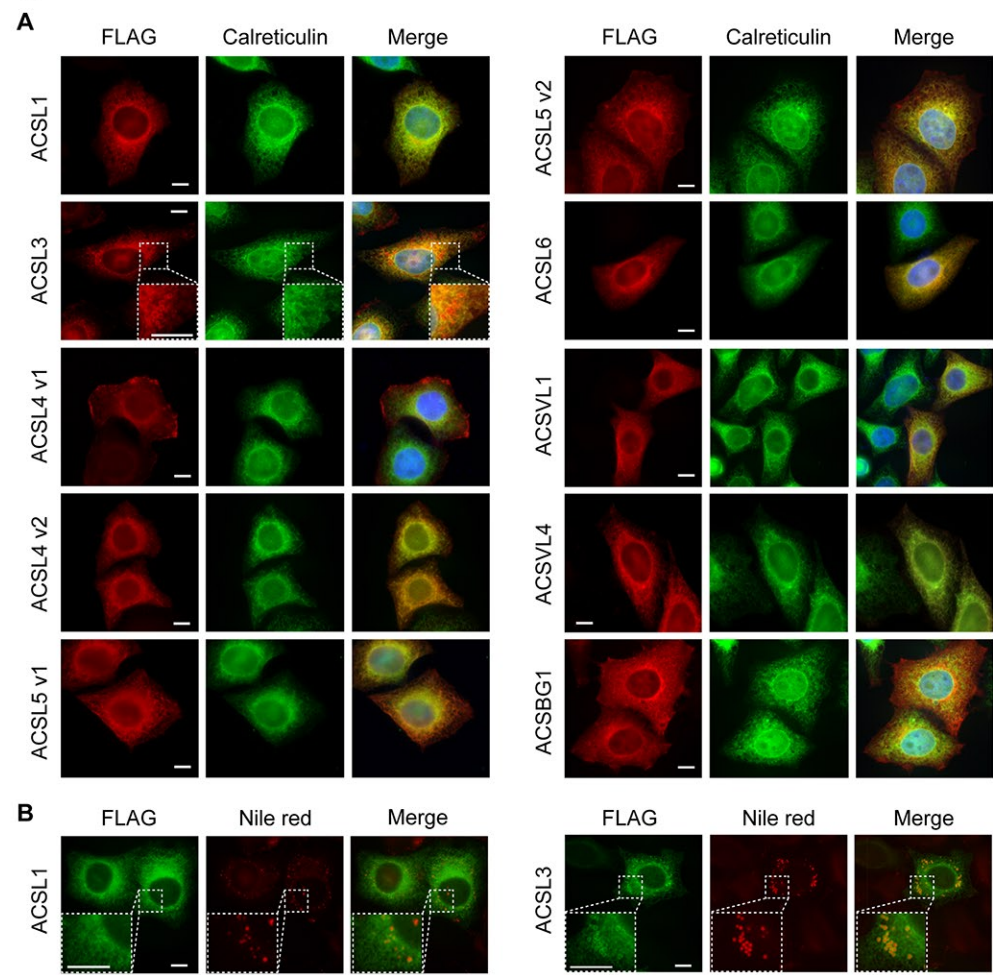


Figure 4

