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Characterization of four mammalian 3-hydroxyacyl-CoA dehydratases involved in very long-chain fatty acid synthesis

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Abstract **Very long-chain fatty acids are produced through a four-step cycle. However, the 3-hydroxyacyl-CoA dehydratase catalyzing the third step in mammals has remained unidentified. Mammals have four candidates, HACD1–4, based on sequence similarities to the recently identified yeast Phs1, although HACD3 and HACD4 share relatively weak similarity. We demonstrate that all four of these human proteins are indeed 3-hydroxyacyl-CoA dehydratases, in growth suppression experiments using a *PHS1*-shut off yeast strain and/or in vitro 3-hydroxypalmitoyl-CoA dehydratase assays. HACD proteins exhibit distinct tissue-expression patterns. We also establish that HACD proteins interact with the condensation enzymes ELOVL1–7, with some preferences.**

Keywords: Very long-chain fatty acid; 3-Hydroxyacyl-CoA dehydratase; Lipid; ELOVL; HACD

1. Introduction

Very long-chain fatty acids (VLCFAs), those having a chain-length $\geqslant 20$, function in cellular processes including maintenance of a functional nuclear envelope [1,2], protein transport [3], and production of bioactive lipid molecules such as eicosanoids [4]. VLCFAs exist not only as free fatty acids (FAs) but also as constituents of other lipids, such as sphingolipids, glycerophospholipids, and glycosylphosphatidylinositol anchors [5,6].

Long-chain FAs are synthesized by FA synthase (FAS) [7]. FA elongation occurs by cycling through a four-step process (condensation, reduction, dehydration, and reduction), with two carbons added through each cycle. Long-chain FAs can be further converted to VLCFAs by ER membrane-bound enzymes cycling through a similar four-step process [8]. Genes encoding enzymes responsible for each step in the latter cycle have been characterized extensively in yeast (condensation, *ELO2*/*FEN1* and *ELO3*/*SUR4*; first reduction, *YBR159w*; dehydration, *PHS1*; and second reduction, *TSC13*) [8,9]. Homology searches led to identification of respective mammalian enzymes: for condensation, ELOVL1–7 (alternative names are listed in Supplementary Table 1) [4]; first reduction, KAR [10]; and second reduction, TER [10]. However, the 3-hydroxyacyl-CoA dehydratase(s) responsible for the dehydration has not been identified.

Yeast Phs1 was recently identified as a 3-hydroxyacyl-CoA dehydratase [8]. This enzyme contains no distinguishing domain or motif, except a C-terminal ER-retention signal. Phs1 is highly enriched with hydrophobic residues and is a six-span membrane protein [11]. The Tyr149 and Glu152 residues of Phs1 are essential for activity and thought to constitute the active site [11].

Mammalian HACD1/PTPLA and HACD2/PTPLB share significant sequence similarities with Phs1 (Supplementary Fig. 1), yet their functions remain unclear. Interactions between HACD2 and BAP31, postulated to be a chaperone or cargo receptor for ER-Golgi transport, have been reported [12], and HACD1 has been linked to certain muscle diseases [13,14]. Other mammalian proteins, HACD3/PTPLAD1/B-ind1 and HACD4/PTPLAD2, share significant sequence similarities with HACD1 and HACD2 and weak similarities with Phs1 (Supplementary Fig. 1). *HACD3* was originally identified as a gene induced by sodium butyrate [15], which inhibits cell proliferation and induces differentiation. HACD3 reportedly interacts with the small GTPase Rac and appears to be involved in Rac1 signaling [15], yet its exact function has remained unclear.

In the study presented here, we characterized these four HACD proteins. We found that all are localized in the ER, and each exhibits a characteristic tissue-expression pattern. Moreover, we demonstrated that all four are 3-hydroxyacyl-CoA dehydratases. We also established that the HACD proteins interact with ELOVL proteins. Our analyses have completed the identification of mammalian enzymes responsible for the entire VLCFA elongation cycle.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cells and HEK 293T cells were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum and supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. HEK 293T cells were grown in 0.3% collagen-coated dishes. Transfections were performed using Lipofectamine Plus™

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Abbreviations: VLCFA, very long-chain fatty acid; FA, fatty acid; SC, synthetic complete; EGFP, enhanced green fluorescent protein

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Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Saccharomyces cerevisiae strains SAY31 and SAY32 [11] were grown in synthetic complete (SC) medium (0.67% yeast nitrogen base and 2% D-glucose) containing nutritional supplements.

2.2. Plasmids

Plasmids constructed in this study are listed in Supplementary Table 2. The pUG34 plasmid, a yeast expression vector constructed to produce a fusion protein with an N-terminal enhanced green fluorescent protein (EGFP), was gift from J.H. Hegemann. The pCE-puro 3xFLAG-1, pCE-puro HA-1, and the pCE-puro His_6-Myc-1 vectors were designed to produce an N-terminal 3xFLAG-tagged protein, an N-terminal HA-tagged protein, and an N-terminal tandemly oriented His_6 and Myc epitope (His_6-Myc)-tagged protein, respectively. The *PHS1*, *HACD1-4*, and *ELOVL1-7* genes were amplified by PCR using primers and templates (Supplementary Tables 3 and 4), and the resulting sequences were verified and cloned into the pUG34, pCE-puro 3xFLAG-1, pCE-puro HA-1, or pCE-puro His_6-Myc-1 vector.

2.3. Immunological assays

Immunoblotting was performed as described previously [16], using the anti-Myc antibody PL-14 (1 μg/ml; Medical and Biological Laboratories, Nagoya, Japan) and anti-GFP antibodies (1 μg/ml; Roche Diagnostics, Indianapolis, IN). Microscopic immunofluorescence studies were performed with anti-Myc PL14 (5 μg/ml) and anti-calreticulin (5 μg/ml; Alexis, Lausen, Switzerland) antibodies [17].

2.4. In vitro 3-hydroxyacyl-CoA dehydratase assays

Using anti-FLAG M2 agarose (Sigma), 3xFLAG-tagged proteins were purified from HeLa cells transfected with the pCE-puro 3xFLAG-HACD1, 2, 3, or 4 plasmid [11]. In vitro 3-hydroxyacyl-CoA dehydratase assays were performed in reaction buffer (50 mM HEPES–NaOH (pH 7.4), 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture (Complete™ EDTA free; Roche Diagnostics), 0.05% digitonin, and 1 mM $MgCl_2$) by incubating the purified proteins with 0.01 μCi [^{14}C]3-hydroxypalmitoyl-CoA (55 mCi/mmol; American Radiolabeled Chemicals, St. Louis, MO) for 15 min at 37 °C [11].

2.5. [^{3}H]Dihydrosphingosine labeling

[^{3}H]Dihydrosphingosine labeling assay was described previously [18].

2.6. Northern blotting

Northern blotting was performed using poly(A)$^+$ RNA blots (Clontech (TAKARA Bio), Palo Alto, CA) containing 1 μg of RNA from 12 different human tissues [17].

3. Results

3.1. HACD1, 2, 3, and 4 proteins are mammalian 3-hydroxyacyl-CoA dehydratases

Phs1 was recently identified as a 3-hydroxyacyl-CoA dehydratase in yeast [8]. However, corresponding mammalian enzyme(s) remained unidentified. Mammals have two proteins that exhibit high sequence similarity to Phs1, PTPLA and PTPLB (identity 24.1% and 26.9%; similarity 39.5% and 45.8%, respectively; Supplementary Fig. 1). Two others exhibit weak but significant sequence similarity to Phs1, PTPLAD1 and PTPLAD2 (identity 15.3% and 19.2%; similarity 29.2% and 39.3%, respectively; Supplementary Fig. 1). Although all contain sequences similar to a protein tyrosine phosphatase motif (HCXXGXXRS/T), none match completely (Supplementary Fig. 1), and so are not expected to possess tyrosine phosphatase activity. Indeed, PTPLB reportedly exhibited no tyrosine phosphatase activity [12]. To reflect their relatedness and, later, their functions, we renamed these proteins HACD1–4 (3-hydroxyacyl-CoA dehydratase; HACD1/PTPLA, HACD2/PTPLB, HACD3/PTPLAD1, and HACD4/PTPLAD2). None exhibits a specific domain, although HACD3 contains a sequence similar to p23/cPGED, a co-chaperone for Hsp90 and/or cytosolic glutathione-dependent prostaglandin E_2 synthase [19,20].

VLCFA synthesis occurs in the ER. Since only HACD2 has been determined to be an ER protein [12], we investigated the intracellular localization of the other proteins. HeLa cells were transfected with plasmids encoding HACD1–4, each tagged with Myc at its N-terminus, and expression was confirmed by immunoblotting with an anti-Myc antibody (Fig. 1A). Indirect immunofluorescence microscopy using the anti-Myc antibody detected all the proteins as reticular structures, with patterns similar to that for ER staining (Fig. 1B). Double-staining of the cells with the anti-Myc antibody and one directed to the ER protein calreticulin confirmed that all HACD proteins are ER-resident (Fig. 1B).

To investigate whether the proteins are functional homologs of Phs1, HACD1–4 were expressed in yeast as EGFP-fused proteins. Since the essential *PHS1* gene cannot be deleted, we used the SAY32 yeast strain, which carries the *PHS1* gene under control of the $TetO_7$ promoter [11]. SAY32 cells containing a vector plasmid were unable to form colonies on SC plates containing doxycyclin (Fig. 2A). When introduced into SAY32 cells, *HACD1* and *HACD2* genes restored growth of the defective cells on doxycyclin-containing plates (Fig. 2A), indicating that they are indeed functional homologs of Phs1, i.e. 3-hydroxyacyl-CoA dehydratases. Neither HACD3 nor HACD4 restored the growth (Fig. 2A). However, in yeast ectopically expressed proteins are often unable to be properly expressed or folded, so an inability to restore growth cannot exclude the possibility that either of these is a 3-hydroxyacyl-CoA dehydratase. Indeed, in immunoblots using anti-GFP antibodies only EGFP-HACD1 was detected near its predicted molecular weight of 59.8 kDa (Fig. 2B). EGFP-HACD2 and EGFP-HACD3 migrated much faster than their predicted molecular weights (55.8 and 70.6 kDa), respectively, and EGFP-HACD3 ran even faster than EGFP (Fig. 2B). Two faint bands were observed for EGFP-HACD4, but again both migrated faster than their predicted molecular weight (54.9 kDa) (Fig. 2B). The faster mobilities may be due to cleavage by a protease or to insufficient denaturing by SDS during the assay, which is often seen with multi-spanning membrane proteins. If a protease is involved, though, some EGFP-HACD2 molecules might remain uncleaved, or the cleaved protein might retain partial activity, since EGFP-HACD2 was able to restore the growth defect of SAY32 cells.

We confirmed the abilities of HACD1 and HACD2 to restore defective Phs1 function in SAY32 cells by examining sphingolipid synthesis. In yeast, ceramide, the backbone of sphingolipids, comprises a C26 VLCFA, and defects in VLCFA synthesis result in accumulations of short-chain ceramide and reductions in complex sphingolipids [11]. Introducing the *HACD1* gene into SAY32 cells completely restored sphingolipid synthesis, similar to *PHS1* results (Fig. 2C). *HACD2* also restored sphingolipid synthesis, but only partially (Fig. 2C). However, again activities in yeast do not always correspond to natural activities in mammalian cells. In conclusion, HACD1 and HACD2 are functional homologs of Phs1,

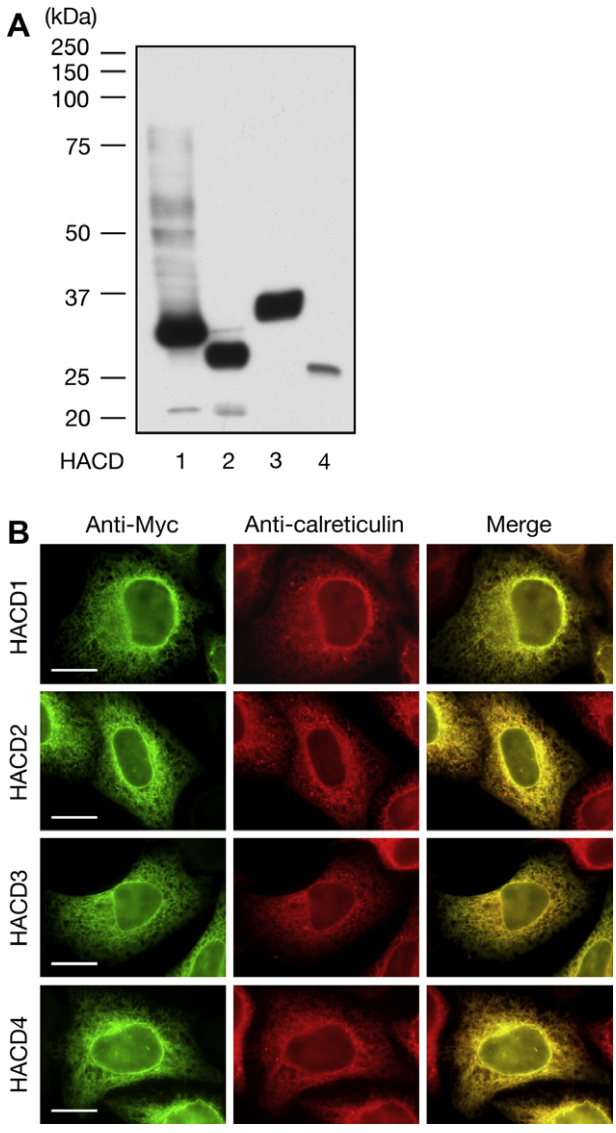

Fig. 1. All HACD proteins are localized in the ER. HeLa cells were transfected with pCE-puro His_6-Myc-HACD1–4. (A) Total cell lysates were prepared and subjected to immunoblotting with an anti-Myc antibody. (B) Cells were fixed with formaldehyde and permeabilized with 0.5% Triton X-100. The cells were then double stained with anti-Myc (left panels) and anti-calreticulin (middle panels) antibodies and analyzed using a fluorescence microscope Axioskop 2 PLUS. Merged images are presented in the right panels. Calibration bar, 20 μm.

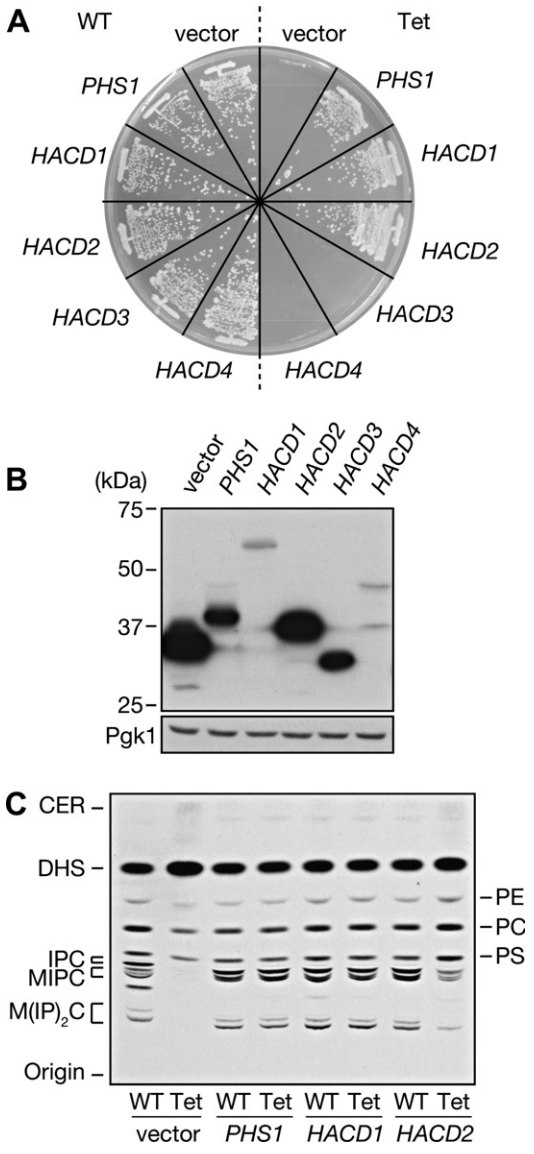

Fig. 2. HACD1 and HACD2 are functional homologs of Phs1. SAY31 (wild type; WT) and SAY32 (*pTetO$_7$-PHS1*; Tet) cells were transfected with the pUG34 (*EGFP* vector), pAK856 (*EGFP-PHS1*), pSH9 (*EGFP-HACD1*), pSH12 (*EGFP-HACD2*), pIKD453 (*EGFP-HACD3*), or pKN27 (*EGFP-HACD4*) plasmid. (A) Cells were grown for 2 days at 30 °C on SC plates lacking histidine and methionine but containing 10 μg/ml doxycyclin. (B) Total cell lysates were prepared and subjected to immunoblotting with an anti-GFP antibody (upper panel) or, to demonstrate uniform protein loading, an anti-Pgk1 antibody (lower panel). (C) Cells were grown for 5 h in 10 μg/ml doxycyclin in SC medium lacking histidine and methionine, then labeled with [^{3}H]dihydrosphingosine for 2 h at 30 °C. Lipids were extracted from the cells and separated by TLC. CER, ceramide; DHS, dihydrosphingosine; IPC, inositolphosphorylceramide; MIPC, mannosylinositol phosphorylceramide; M(IP)$_2$C, mannosyldiinositol phosphorylceramide; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine.

although equivalent functions were not established for HACD3 or HACD4 by these assays.

To prove directly that HACD1 and HACD2 are 3-hydroxyacyl-CoA dehydratases and to investigate whether HACD3 and HACD4 also exhibit such activity, we performed in vitro assays using affinity-purified HACD proteins tagged with 3xFLAG and [^{14}C]3-hydroxypalmitoyl-CoA as a substrate. All the proteins converted 3-hydroxypalmitoyl-CoA to 2,3-*trans* hexadecenoyl-CoA (Fig. 3A). By changing concentrations of the substrate we found that each enzyme exhibited distinct K_m and V_{max} values. HACD1 and HACD3 exhibited similar, intermediate values (K_m = 33.6 μM, V_{max} = 49.3 μM and K_m = 49.5 μM, V_{max} = 65.8 μM, respectively) (Fig. 3B). HACD2 had comparatively larger values (K_m = 121.7 μM, V_{max} = 181.8 μM), whereas HACD4 had smaller (K_m = 6.8 μM, V_{max} = 11.6 μM) (Fig. 3B). Thus, HACD2 exhibited a lower affinity for 3-hydroxypalmitoyl-CoA but a higher reaction velocity, and HACD4 exhibited a higher affinity but lower reaction velocity.

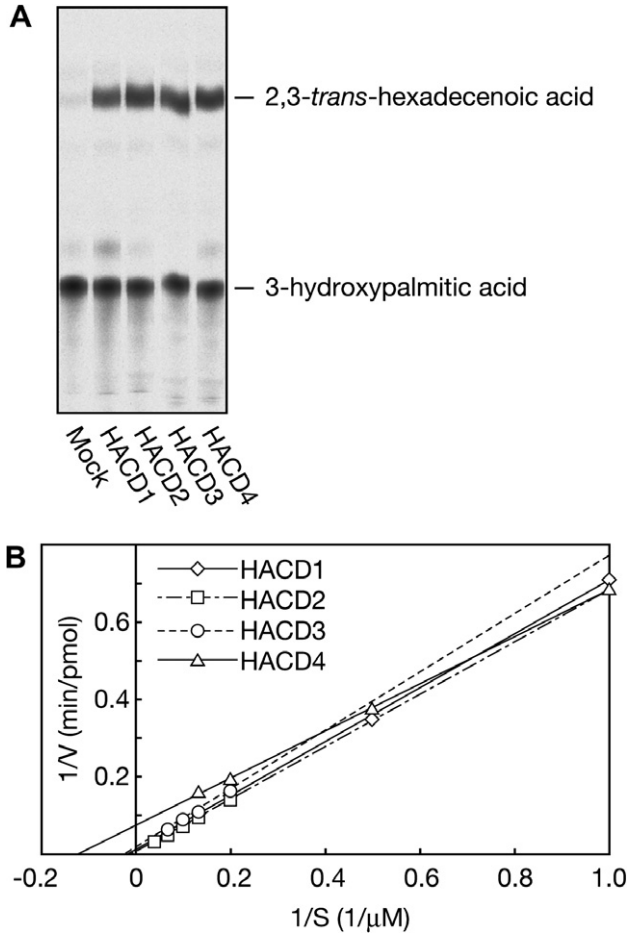

Fig. 3. All HACD proteins exhibit 3-hydroxyacyl-CoA dehydratase activity. HeLa cells were transfected with pCE-puro 3xFLAG-1 vector (mock) or pCE-puro 3xFLAG plasmids encoding 3xFLAG-HACD1, 2, 3, and 4. Proteins were purified using anti-FLAG M2 beads, and samples (20 ng protein) were incubated for 15 min at 37 °C with [^{14}C]3-hydroxypalmitoyl-CoA (A, total 5 μM; B, 1–25 μM as indicated). After termination of the reactions, lipids were saponified, acidified, extracted, and separated by TLC. (B) The radioactivities associated with the reaction product 2,3-*trans* hexadecenoic acid were quantified using a bioimaging analyzer BAS-2500 and are expressed in a Lineweaver–Burg plot.

3.2. HACD proteins exhibit distinct expression patterns in human tissues

Expression of human *HACD1* mRNA is known to be restricted to heart and (weakly) skeletal muscle [13], whereas *HACD2* mRNA is ubiquitously expressed [12]. Tissue-expression patterns have not previously been investigated for human *HACD3* or 4 mRNA, although in mouse, *HACD3* mRNA was determined to be expressed nearly ubiquitously [15]. We performed high stringency Northern blot analysis using mRNAs extracted from various human tissues. A predominant 3.2 kb *HACD3* mRNA was detected in most tissues examined, except leukocytes (Fig. 4). The highest expression was observed in brain, kidney, and liver, but only low levels were detected in skeletal muscle. In contrast, expression of *HACD4* mRNA was highly tissue-specific. High expression was observed only in leukocytes, and low expression in heart, spleen, kidney, placenta, and lung (Fig. 4).

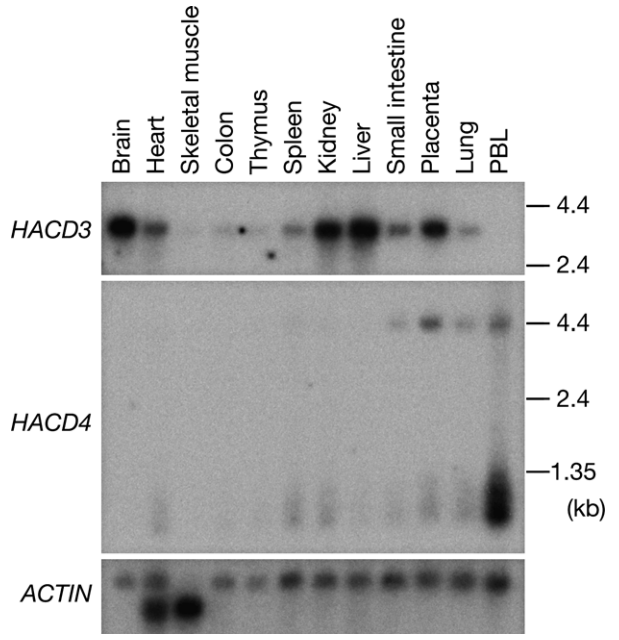

Fig. 4. *HACD3* and *HACD4* mRNAs exhibit different tissue-specific expression patterns. ^{32}P-labeled *HACD3*, *HACD4*, and *actin* probes were hybridized to poly(A)$^+$ RNA blots containing 1 μg of RNA from the indicated human tissues. PBL, peripheral blood leukocytes.

3.3. HACD proteins interact with ELOVL proteins with some preferences

In yeast, enzymes involved in the VLCFA elongation cycle form an elongase complex [8]. Mammals have seven condensation enzymes (ELOVL1–7) and four 3-hydroxyacyl-CoA dehydratases (HACD1–4). However, it is unclear whether mammalian enzymes also form elongase complex(es) or if each HACD protein interacts with specific ELOVL protein(s). To address these questions, we performed co-immunoprecipitation experiments using anti-FLAG antibodies and lysates prepared from HEK 293T cells expressing various combinations of a 3xFLAG-tagged HACD protein and a HA-tagged ELOVL protein. Interactions were observed for all sets of co-expressed HACD-FLAG and ELOVL-HA proteins (Fig. 5), although ELOVL1 bands were difficult to see due to low expression. The strength of the interactions varied. ELOVL3 and ELOVL6 interacted with HACD1 and HACD4 more strongly than with HACD2 and HACD3. Interestingly, HACD3 exhibited an apparently higher affinity for ELOVL2 and ELOVL7, since, despite their lower or equivalent expression levels, the amounts of ELOVL2 and ELOVL7 in the precipitated fractions were similar to those of ELOVL3, ELOVL5, and ELOVL6, and greater than that of ELOVL4 (Fig. 5). These results suggest that ELOVLs have the potential to function with any HACD protein, although some preferences exist.

4. Discussion

VLCFA synthesis occurs by cycling through a four-step process. The enzyme catalyzing the third step, a 3-hydroxyacyl-CoA dehydratase, had not been identified in mammals. Phs1 was recently identified as the yeast 3-hydroxyacyl-CoA dehy-

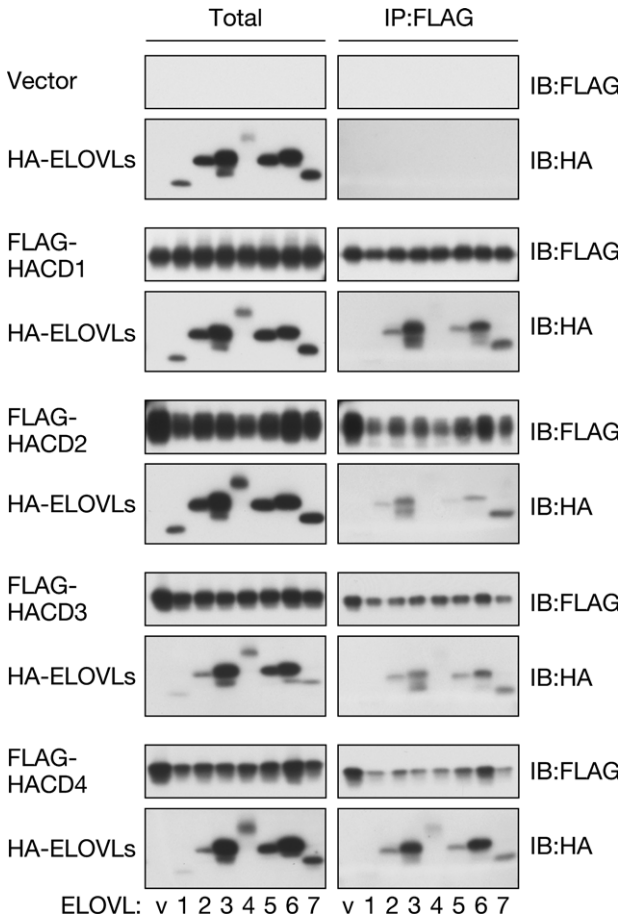

Fig. 5. HACD proteins interact with ELOVL proteins with some preferences. HEK 293T cells were transfected with the pCE-puro HA-1 control vector or pCE-puro HA-ELOVLx plasmids (where x is each ELOVL number), and with pCE-puro 3xFLAG-HACDx plamids (where x is each HACD number) or the pCE-puro 3xFLAG-1 control vector. Total cell lysates were then prepared from the cells and solubilized with 1% Triton X-100. Following immunoprecipitation with anti-FLAG M2 agarose, total lysates (1/4 amount) and bound proteins were subjected to immunoblotting with anti-FLAG or anti-HA antibodies. IP, immunoprecipitation; IB, immunoblotting.

dratase [8], and a homology search revealed that Phs1 is conserved among eukaryotes. Mammals have two apparent homologs of Phs1 (HACD1 and HACD2) and two proteins sharing weak but significant homology (HACD3 and HACD4) (Supplementary Fig. 1). In the presented study, we revealed that all these HACD proteins are indeed mammalian 3-hydroxyacyl-CoA dehydratases. HACD1 and HACD2 restored the growth defect of a *pTetO$_7$-PHS1* yeast strain (Fig. 2A). In vitro analyses demonstrated that HACD1, 2, 3, and 4 all exhibited 3-hydroxypalmitoyl-CoA dehydratase activities, although their K_m and V_{max} values differed greatly (Fig. 3B).

Although yeast contains only one 3-hydroxyacyl-CoA dehydratase Phs1, mammals have four isozymes. Therefore, it was expected that each HACD protein would have a specific function. One particular difference is in their tissue-expression patterns (Fig. 4) [12,13,15]. Moreover, the HACD proteins exhibited certain preferences in interacting with ELOVL proteins, although strict specificities were not observed (Fig. 5). It is possible, however, that each HACD protein exhibits preference(s) toward certain 3-hydroxyacyl-CoAs, such as long-chain, very long-chain, saturated, or unsaturated 3-hydroxyacyl-CoAs, since we used only 3-hydroxypalmitoyl-CoA as a substrate due to limited commercial availability.

The expression of HACD2 and HACD3 is ubiquitous (Fig. 4) [12], whereas HACD1 and HACD4 expression is restricted to certain tissues (HACD1, heart; HACD4, leukocytes; Fig. 4) [13]. Likewise, ELOVL1, ELOVL5, and ELOVL6 are ubiquitously expressed, whereas ELOVL2, ELOVL3, and ELOVL4 are tissue-specific (ELOVL2, liver and testis; ELOVL3, skin and liver; ELOVL4, retina and brain; Supplementary Table 5). Therefore, HACD2 or HACD3 in combination with ELOVL1, ELOVL5, or ELOVL6 may catalyze the synthesis of VLCFAs common to most tissues. On the other hand, HACD1, HACD4, ELOVL2, ELOVL3, ELOVL4, and perhaps ELOVL7, whose expression profile is not known, may function in the production of tissue-specific VLCFAs. Further experiments into these combinations might address these enticing possibilities.

The K_m values of HACD1, 2, 3, and 4 seemed to be much higher than those for expected cellular concentrations of 3-hydroxyacyl-CoAs. However, these values may be caused by in vitro assay conditions. Under physiological conditions the HACD proteins and their 3-hydroxyacyl-CoAs substrates are embedded in the ER membrane, whereas the in vitro HACD proteins and substrates were solubilized with detergent, and so existed in micelles. Access of the HACD proteins to substrates in different micelles would be achieved only by fusion of the micelles. Therefore, the K_m values would be undervalued compared to those under physiological conditions. We speculate that even the physiological K_m of HACD2, which exhibited the highest of the four proteins, is much lower than for the cellular concentrations of 3-hydroxyacyl-CoA. If this is true, HACD2 contributes to the dehydration of, at least, 3-hydroxy long-chain saturated acyl-CoA such as 3-hydroxypalmitoyl-CoA in cells, since it exhibited the highest V_{max} value toward 3-hydroxypalmitoyl-CoA (Fig. 3B). Our preliminary results of an in vitro elongase assay using membrane fractions from siRNA-treated HeLa cells with [^{14}C]malonyl-CoA and cold palmitoyl-CoA have indicated that only siRNA for *HACD2* causes an accumulation of 3-hydroxystearoyl-CoA. However, future studies are required to determine the substrate specificity of each HACD protein.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.06.007.

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