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The Human Liver-specific Homolog of Very Long-chain Acyl-CoA Synthetase Is Cholate:CoA Ligase*

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Unconjugated bile acids must be activated to their CoA thioesters before conjugation to taurine or glycine can occur. A human homolog of very long-chain acyl-CoA synthetase, hVLCS-H2, has two requisite properties of a bile acid:CoA ligase, liver specificity and an endoplasmic reticulum subcellular localization. We investigated the ability of this enzyme to activate the primary bile acid, cholic acid, to its CoA derivative. When expressed in COS-1 cells, hVLCS-H2 exhibited cholate:CoA ligase (choloyl-CoA synthetase) activity with both non-isotopic and radioactive assays. Other long- and very long-chain acyl-CoA synthetases were incapable of activating cholate. Endogenous choloyl-CoA synthetase activity was also detected in liver-derived HepG2 cells but not in kidney-derived COS-1 cells. Our results are consistent with a role for hVLCS-H2 in the re-activation and re-conjugation of bile acids entering liver from the enterohepatic circulation rather than in *de novo* bile acid synthesis.

Synthesis of bile acids from cholesterol and their conversion to bile salts (conjugated bile acids) takes place in the liver (reviewed in Refs. 1 and 2). The primary bile acids synthesized in humans are cholic acid and chenodeoxycholic acid. These compounds are conjugated to the amino acids glycine or taurine, which are then secreted into the bile canaliculi. For conjugation, bile acids must first be activated to their coenzyme A (CoA) thioesters. This activation is catalyzed by an acyl-CoA synthetase (bile acid:CoA ligase) that is distinct from the enzyme(s) that activate long-chain fatty acids (3, 4); however, the bile acid ligase reaction mechanism is analogous to that of other acyl-CoA synthetases (5). Whereas long-chain fatty acid

activation occurs in essentially all tissues, bile acid activation takes place only in liver (3). Subcellular fractionation studies revealed that activation occurred in the endoplasmic reticulum of liver (3, 6–8).

Our laboratory recently identified a family of human proteins homologous to very long-chain acyl-CoA synthetase (VLCS)¹ (9–11), an enzyme originally purified from rat liver peroxisomes (12). Unlike long-chain acyl-CoA synthetase (LCS), VLCSs from both rat (rVLCS) and human (hVLCS) are capable of activating fatty acids containing more than 22 carbons (11, 12). One protein, which we previously designated human VLCS homolog 2 (hVLCS-H2), possessed the two criteria necessary for a bile acyl-CoA synthetase, a liver-specific tissue distribution and an endoplasmic reticulum subcellular localization (10). Therefore, we investigated the ability of this enzyme to activate cholic acid.

EXPERIMENTAL PROCEDURES

Materials and General Methods—HepG2 cells were obtained from ATCC (HB-8065). COS-1 cells were a gift from Dr. C. Thompson. [1-¹⁴C]Palmitic acid and [1-¹⁴C]cholic acid were obtained from American Radiolabeled Chemicals. [1-¹⁴C]Lignoceric acid was synthesized from tricosanol and K¹⁴CN (American Radiolabeled Chemicals) by the method of Muralidharan and Kishimoto (13). Choloyl-CoA was synthesized by the mixed anhydride method as described by Schulz (14). Protein was determined by the method of Lowry *et al.* (15).

Acyl-CoA Synthetase cDNAs, Transfection of COS-1 Cells, and Assay of Fatty Acyl-CoA Synthetase Activity—Full-length cDNA encoding human VLCS (hVLCS), hVLCS-H2, and a rat LCS (rLCS1) were obtained and cloned into the mammalian expression vector pcDNA3 (Invitrogen) as described previously (10, 11, 16). Details of hVLCS homolog 1 (hVLCS-H1) will be published elsewhere.² COS-1 cells were transiently transfected with full-length cDNA encoding each acyl-CoA synthetase or with the pcDNA3 vector alone by electroporation at 250 V as described previously (11). Three days post-transfection, cells were harvested by trypsinization, washed with phosphate-buffered saline, and stored at –80 °C prior to assay. Assay of activation of [1-¹⁴C]fatty acids was performed as described previously (17).

Choloyl-CoA Synthetase Assays—Two assays of choloyl-CoA synthetase were used. Non-isotopic assays contained 40 mM Tris, pH 7.5, 10 mM ATP, 5 mM MgCl₂, 0.2 mM CoA, 20 μM cholic acid (sodium salt), and COS-1 cells transiently expressing acyl-CoA synthetases (40 μg protein) in a total volume of 0.25 ml. After 20 min at 37 °C, duplicate 100-μl aliquots were removed and the reaction was stopped by addition of 10 μl of 2 N HCl and 12.5 μl of 0.6 M MES in 2 N KOH. Samples were stored at –20 °C. Just prior to analysis, samples were thawed and 47 μl of acetonitrile was added. HPLC analysis was performed using a modification of the method of Kurosawa *et al.* (18). Samples were injected onto a Phenomenex 15-cm, 3-μm C-18 reverse-phase HPLC column. Bile acyl-CoAs were eluted using a gradient of acetonitrile in 16.9 mM sodium phosphate, pH 6.9 (25–37% over 20 min). Absorbance at 254 nm was monitored. For the radioactive assay, 20 μM [1-¹⁴C]cholic acid (20,000 dpm/nmol) replaced the unlabeled compound. Labeled choloyl-CoA was separated from the substrate by a modification of the method of Kelley and Vessey (5). The reaction was terminated by the addition of 0.05 ml of 0.1 M EDTA, pH 7.5, and 0.2 ml of 60 mM succinic acid. Unreacted cholate was extracted with two 1.0-ml portions of water-saturated butanol. Radioactivity in the aqueous phase containing choloyl-CoA was determined.

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¹ The abbreviations used are: VLCS, very long-chain acyl-CoA synthetase; LCS, long-chain acyl-CoA synthetase, rVLCS, rat VLCS; hVLCS, human VLCS; hVLCS-H, human VLCS homolog; MES, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography; FATP, fatty acid transport protein; THCA, trihydroxycholestanic acid; DHCA, dihydroxycholestanic acid.

² S. J. Steinberg and P. A. Watkins, manuscript in preparation.

TABLE I
Tissue distribution and subcellular localization of VLCS/FATP proteins

The nomenclature proposed by Hirsh *et al.* (25) for hVLCS, hVLCS-H1, and hVLCS-H2 is shown in parentheses in the first column. e. r., endoplasmic reticulum.

	Subcellular localization	Tissue distribution	References
hVLCS (FATP2)	peroxisomes, e. r.	liver, kidney, heart, brain, and fibroblasts	11
hVLCS-H1 (FATP6)	e. r.	heart	2
hVLCS-H2 (FATP5)	e. r.	liver	10
mFATP1	plasma membrane	adipose, brain, heart, skeletal muscle, and kidney	25, 26
mFATP4	plasma membrane	small intestine, liver, kidney, and brain	25, 27

RESULTS AND DISCUSSION

hVLCS-H2 Fulfills the Criteria Necessary for a Bile Acyl-CoA Synthetase—It was hypothesized more than 20 years ago that the enzyme that activates bile acids to their CoA derivatives is distinct from LCS, the enzyme that activates the most abundant endogenous and dietary fatty acids (3). This hypothesis was based primarily on the observation that whereas microsomes from nearly all tissues activated 16- and 18-carbon fatty acids, only those from liver were capable of activating cholic acid (3). Furthermore, activation of fatty acids takes place in mitochondria and peroxisomes, as well as in microsomes (19), whereas cholate activation is almost exclusively microsomal (3, 6–8). Thus, the two criteria that a bile acyl-CoA synthetase must fulfill are liver specificity and a microsomal subcellular location.

Our investigations of a new family of proteins that includes VLCS and fatty acid transport protein (FATP) revealed that a member of this family, hVLCS-H2, possessed these two attributes (Table I) (10). We recently reported cloning of full-length hVLCS-H2 cDNA and initial characterization of its gene product (10). When an hVLCS-H2-specific probe derived from this clone was hybridized to a human multiple tissue Northern blot, a transcript was detected only in liver (10). Furthermore, hVLCS-H2 containing the *c-myc* epitope at its N terminus was found localized to the endoplasmic reticulum in either COS-1 cells or HepG2 cells transiently expressing the protein (10). When the acyl-CoA synthetase activity of COS-1 cells expressing hVLCS-H2 was assessed, the enzyme weakly activated fatty acids with chain length acids from 18 to 26 carbons. However, hVLCS-H2 was not nearly as robust at activating either long- or very long-chain fatty acids as hVLCS, an enzyme that is also present in liver endoplasmic reticulum (11). Therefore, we questioned whether the endogenous substrate for hVLCS-H2 might be a liver-specific fatty acyl moiety such as cholic acid.

Amino acid sequence comparisons also supported hVLCS-H2 as a candidate for choloyl-CoA synthetase. Mallonee *et al.* (20) previously reported that the *baiB* gene from *Eubacterium* sp. encoded a bile acid:CoA ligase. The deduced amino acid sequence of hVLCS-H2 exhibited 26% amino acid identity and 48% similarity to the *baiB* gene product. Furthermore, the bacterial ligase contains a motif that we previously identified as unique to the VLCS family (Fig. 1) (9).

hVLCS-H2 Activates Cholic Acid—To determine whether hVLCS-H2 could activate cholic acid to its CoA derivative, COS-1 cells transiently expressing this enzyme were incubated with cholic acid in the presence of ATP/Mg²⁺ and CoA. Reaction products were separated by reverse-phase HPLC as described under “Experimental Procedures.” A single peak eluting at 16.2 min was observed (Fig. 2). This peak was not formed when reaction mixtures lacked ATP, CoA, or cholate, suggesting that the peak is choloyl-CoA (data not shown). The retention time of this peak was similar to that reported for authentic choloyl-CoA chromatographed under similar conditions (18). To verify that the reaction product was truly choloyl-CoA, we added a known quantity of authentic choloyl-CoA to hVLCS-

hVLCS	475	IYFHDRVGDTRFKGENVATIEVA	498
hVLCS-H1	474	LYFDRITGDTRFKGENVATIEVA	497
hVLCS-H2	545	LYFDRITGDTRFKGENVSTIEVE	568
BaiBp	411	LYESDRSMLVSGGENVFATIEVE	434
mFATP1	502	MYFRDRSGDTRFKGENVSTIEVE	525
mFATP4	498	LYFRDRITGDTRFKGENVSTIEVE	521

FIG. 1. Bacterial bile acid:CoA ligase and hVLCS-H2 share a conserved motif. We previously described a motif unique to the VLCS/FATP protein family (9). 17 of 24 residues in this region are identical in the mammalian proteins (**bold**). This motif was also present in the bacterial protein encoded by the *baiB* gene, a bile acid:CoA ligase. 13 of 24 amino acids of BaiB protein and hVLCS-H2 are identical (*shaded*). mFATP, murine FATP.

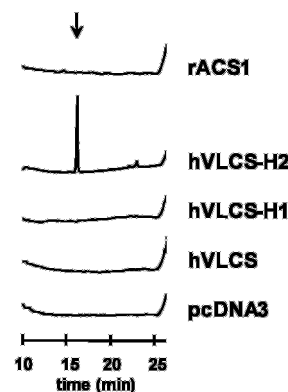


FIG. 2. Choloyl-CoA synthetase activity of transfected COS-1 cells. COS-1 cells were transfected with the indicated plasmid. After 72 h, cells were harvested, stored at -80°C , and incubated with cholic acid, and the products were analyzed by reverse-phase HPLC as described under “Experimental Procedures.” Analysis of samples from two independent transfection experiments yielded identical results.

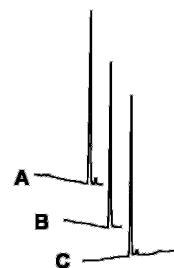


FIG. 3. Identification of the hVLCS-H2 reaction product as choloyl-CoA. A, authentic choloyl-CoA was chromatographed on a reverse-phase HPLC column as described under “Experimental Procedures.” B, HPLC analysis of reaction products formed when COS-1 cells transiently expressing hVLCS-H2 were incubated with cholic acid as described in the legend to Fig. 2. C, HPLC analysis of a 50:50 mixture of the samples shown in A and B.

H2-containing reaction mixtures prior to injection onto the HPLC column and observed a single peak on chromatograms (Fig. 3).

COS-1 cells transfected with the pcDNA3 vector alone showed little capacity to activate cholate (Fig. 2). This result is consistent with a lack of endogenous choloyl-CoA synthetase in these cells that were originally derived from monkey kidney.

TABLE II

Activation of [$1\text{-}^{14}\text{C}$]fatty acids or [$1\text{-}^{14}\text{C}$]cholate by COS-1 cells transiently expressing acyl-CoA synthetases

COS-1 cells were transiently transfected with the indicated plasmid. Three days post-transfection, cells were harvested, frozen at -80°C , and subsequently assayed for their ability to activate palmitic acid (C16:0), lignoceric acid (C24:0), or cholic acid as described under "Experimental Procedures." Results are the average of three independent transfection experiments \pm S.D. Compared with endogenous (pcDNA3-transfected COS-1 cell) activity, hVLCs activated both C16:0 and C24:0. hVLCs-H2, weakly activated C24:0, whereas rACS1 primarily activated C16:0. These activities are consistent with our previous observations (10, 11). The natural substrate for hVLCs-H1 remains uncertain, although COS-1 cells stably expressing this protein weakly activate C24:0.²

	COS-1 cells expressing				
	pcDNA3	hVLCs	hVLCs-H1	hVLCs-H2	rACS1
	nmol/20 min/mg of protein				
Activation of					
C16:0	55.4 \pm 8.6	137 \pm 50	39.2 \pm 7.7	51.0 \pm 10.4	157 \pm 21
C24:0	0.83 \pm 0.16	8.57 \pm 6.4	0.84 \pm 0.28	1.63 \pm 0.55	1.13 \pm 0.18
Cholate	0.01 \pm 0.01	0.01 \pm 0.01	<0.01	1.98 \pm 0.85	<0.01

Furthermore, hVLCs, the heart-specific hVLCs-H1, and rat LCS (rACS1) were all incapable of activating cholic acid when expressed in COS-1 cells (Fig. 2). In contrast, each of these expressed proteins showed enzymatic activity with their appropriate long- or very long-chain fatty acyl substrates (Table II).

Preference of hVLCs-H2 for Cholic Acid—To assess more thoroughly the substrate preference of hVLCs-H2 for cholic acid, acyl-CoA synthetase assays using [$1\text{-}^{14}\text{C}$]cholic acid as substrate were performed. As shown in Table II, COS-1 cells expressing hVLCs-H2 activated cholic acid to its CoA derivative. Cells expressing vector alone, hVLCs, hVLCs-H1, or rACS1 had essentially no detectable choloyl-CoA synthetase activity, as predicted from the non-isotopic assay. To compare increases in acyl-CoA synthetase activities due to expression of the different enzymes, the endogenous activity of COS-1 cells (cells transfected with pcDNA3 alone) was subtracted. The hVLCs-H2-specific increase in choloyl-CoA synthetase was more than 2-fold higher than the increase in C24:0 synthetase (1.97 versus 0.80 nmol/hr/mg of protein), suggesting that cholic acid, rather than very long-chain fatty acid, was the preferred substrate of hVLCs-H2.

Choloyl-CoA Synthetase Activity in HepG2 Cells—We previously showed by reverse-transcription PCR that hVLCs-H2 message was present in the human hepatoma-derived cell line, HepG2, whereas no message was detected in skin fibroblasts, heart or brain (10). To verify that HepG2 cells were capable of activating cholic acid, these cells were incubated with [$1\text{-}^{14}\text{C}$]cholic acid. Endogenous cholate synthetase activity of HepG2 cells was 7-fold higher than that of COS-1 cells, whereas activation of C16:0 and C24:0 were comparable in the two cell lines (Table III).

Role of Choloyl-CoA Synthetase In Bile Acid Metabolism—Conversion of the C27 sterol, cholesterol, to the C24 bile acids is a multistep process (reviewed in Refs. 1 and 2). Initial reactions include a series of ring modifications including hydroxylations, saturation of the ring system, and epimerization of the 3- β -hydroxyl group. One of the two terminal methyl groups of the side chain is oxidized to a carboxylic acid, yielding trihydroxycholestanic acid (THCA) and dihydroxycholestanic acid (DHCA), the primary intermediates in bile acid synthesis. These synthetic reactions are catalyzed by enzymes in the endoplasmic reticulum, cytosol, and mitochondria. Subsequently, the side chains of THCA and DHCA must be activated to their CoA derivatives, followed by one cycle of β -oxidation in peroxisomes (21). The products of THCA and DHCA β -oxidation are choloyl-CoA and chenodeoxycholoyl-CoA, respectively; propionyl-CoA is released in this process. Conjugation to glycine or taurine, catalyzed by bile acid CoA:amino acid *N*-acyltransferase, has also been reported to occur in peroxisomes (22). Glyco- and tauro-conjugates exit the peroxisome by an unknown mechanism and are secreted into bile canaliculi.

TABLE III

Activation of [$1\text{-}^{14}\text{C}$]fatty acids or [$1\text{-}^{14}\text{C}$]cholate by HepG2 and COS-1 cells

HepG2 or COS-1 cells were grown to confluence, harvested, stored at -80°C , and assayed for their ability to activate the indicated [$1\text{-}^{14}\text{C}$] acid as described under "Experimental Procedures." Results are the average of two independent analyses.

	HepG2	COS-1
	nmol/20 min/mg of protein	
Activation of		
C16:0	55.8	42.2
C24:0	0.64	0.66
Cholate	0.104	0.015

If taurocholate and glycocholate are synthesized *de novo* within peroxisomes, what then is the role of the endoplasmic reticulum protein choloyl-CoA synthetase/hVLCs-H2? About 90% of the bile acids secreted into the small intestine are reabsorbed throughout the small bowel, primarily at the distal ileum (1, 23). Bacterial flora deconjugate and subsequently dehydroxylate a considerable portion of cholic and chenodeoxycholic acids (primary bile acids), yielding the secondary bile acids, deoxycholic and lithocholic acids, respectively (1, 23). Any unconjugated bile acids returning to the liver must be re-activated and re-conjugated. Whereas the highest specific activity of bile acid CoA:amino acid *N*-acyltransferase has been found in peroxisomes, activity is also found in cytosol and endoplasmic reticulum (6, 7, 22). Thus, based on the subcellular localization for hVLCs-H2 and other bile acid-metabolizing enzymes, we hypothesize that the primary role of hVLCs-H2 may be in the re-activation pathway and not the *de novo* biosynthetic pathway. Studies to evaluate this hypothesis are underway. Furthermore, Wheeler *et al.* (24) recently purified a bile acid:CoA ligase from rat liver. It will be interesting to determine whether their enzyme is the rat ortholog of hVLCs-H2.

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