Participation of Two Members of the Very Long-chain Acyl-CoA Synthetase Family in Bile Acid Synthesis and Recycling*

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Bile acids are synthesized de novo in the liver from cholesterol and conjugated to glycine or taurine via a complex series of reactions involving multiple organelles. Bile acids secreted into the small intestine are efficiently reabsorbed and reutilized. Activation by thioesterification to CoA is required at two points in bile acid metabolism. First, 3α,7α,12α-trihydroxy-5β-cholestanolic acid (THCA), the 27-carbon precursor of cholic acid, must be activated to its CoA derivative before side chain cleavage via peroxisomal β-oxidation. Second, reutilization of cholate and other C24 bile acids requires reactivation prior to re-conjugation. We reported previously that homolog 2 of very long-chain acyl-CoA synthetase (VLCS) can activate cholate (Steinberg, S. J., Mihalik, S. J., Kim, D. G., Cuebas, D. A., and Watkins, P. A. (2000) J. Biol. Chem. 275, 15605–15608). We now show that this enzyme also activates chenodeoxycholate, the secondary bile acids deoxycholate and lithocholate, and 3α,7α,12α-trihydroxy-5β-cholestanolic acid. In contrast, VLCS activated 3α,7α,12α-trihydroxy-5β-cholostanoate, but did not utilize any of the C24 bile acids as substrates. We hypothesize that the primary function of homolog 2 is in the reactivation and recycling of C24 bile acids, whereas VLCS participates in the de novo synthesis pathway. Results of in situ hybridization, topographic orientation, and inhibition studies are consistent with the proposed roles of these enzymes in bile acid metabolism.

The synthesis of bile acids from cholesterol is a complex process requiring modifications of both the steroid nucleus and the aliphatic side chain (reviewed in Refs. 1 and 2). Cholic acid and chenodeoxycholic acid, the two primary bile acids in humans, are synthesized in the liver via the concerted action of enzymes located in the endoplasmic reticulum, cytosol, mitochondria, and peroxisomes. The immediate precursors of the C24 bile acids cholate and chenodeoxycholate are the C27 compounds 3α,7α,12α-trihydroxy-5β-cholestanolic acid (THCA) and 3α,7α-dihydroxy-5β-cholestanolic acid (DHCA), respectively (1, 2). It is well established that chain shortening of the methyl-branched side chain of THCA and DHCA by β-oxidation takes place in peroxisomes (3). For β-oxidation to occur, THCA and DHCA must first be activated to their CoA thiosters. The products of chain shortening are choloyl-CoA and chenodeoxycholoyl-CoA and not the free bile acids (4).

The CoA thiosters of the primary bile acids are conjugated to the amino acid glycine or taurine in the hepatocyte before secretion into the bile canaliculi (1, 2). After bile secretion into the intestine during digestion, the conjugated primary bile acids are subject to deconjugation, 7α-dehydroxylation, and other modifications by the intestinal flora (1, 5). 7α-Dehydroxylation converts conjugated or unconjugated cholate to deoxycholate, and chenodeoxycholate to lithocholate. Deoxycholate and lithocholate are referred to as secondary bile acids. A substantial portion of the intestinal bile acid pool is reabsorbed by passive diffusion throughout the small bowel and by active transport in the distal ileum; all of these compounds return to the liver via the enterohepatic circulation for reutilization (6). Recycled unconjugated primary and secondary bile acids must then be re-conjugated to glycine or taurine before return to the bile. However, re-conjugation can occur only after the free acids are activated to their CoA derivatives.

We recently demonstrated that human very long-chain acyl-CoA synthetase homolog 2 (hVLCS-H2), a liver-specific member of the protein family that includes very long-chain acyl-CoA synthetases (VLCSs) and fatty acid transport proteins, is a choloyl-CoA synthetase (7). We hypothesized that a major function of this protein is to activate recycled bile acids prior to their re-conjugation. In this report, we provide further evidence for a role for hVLCS-H2 in activation of primary and secondary bile acids. Furthermore, we demonstrate that hVLCS and hVLCS-H2 are capable of activating the cholic acid precursor (THCA) and speculate on the complementary role of these proteins in bile acid synthesis. Based on the substrate specificity of hVLCS-H2, we will hereafter refer to this protein as human bile acyl-CoA synthetase (hBACS).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF033031.

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1 The abbreviations used are: THCA, 3α,7α,12α-trihydroxy-5β-cholestanolic acid; DHCA, 3α,7α-dihydroxy-5β-cholestanolic acid; hVLCS, human very long-chain acyl-CoA synthetase; hVLCS-H1 and hVLCS-H2, hVLCS homologs 1 and 2, respectively; hBACS, human bile acyl-CoA synthetase; EST, expressed sequence tag; HPLC, high-performance liquid chromatography; rACS1, rat liver long-chain acyl-CoA synthetase-1.
**EXPERIMENTAL PROCEDURES**

**Materials and General Methods**—Sodium salts of cholic, chenodeoxycholic, deoxycholic, and lithocholic acids were obtained from either Steraloids, Inc. (Wilton, NH) or Sigma. [1-4C]Cholic acid (55 mCi/mmol) and [1-4C]chenodeoxycholic (5.13 mCi/mmol) were obtained from American Radiolabeled Chemicals. [1-14C]Palmitic acid (50 mCi/mmol) was from Moravek Biochemicals, Inc. The PCR conditions were as previously described (8). Protein was determined by the method of Lowry et al. (9).

**Synthesis of THCA and [26-14C]THCA—Δ24-THCA was synthesized by the method of Xu and Cuebas (10). Unlabeled THCA was prepared by the catalytic hydrogenation of Δ24-THCA using Adams’ catalyst (Aldrich) dissolved in methanol at 1-atmosphere pressure for 12 h. The product is a racemic mixture of (2S)- and (2R)-THCA stereoisomers. [26-14C]THCA was prepared from THCA by a modification of the method of Tserng and Klein (11). THCA was formulated to protect the hydroxyl groups by heating 0.24 mmol in 1 ml of 90% formic acid containing 0.025 ml of 70% perchloric acid for 2 h at 55 °C. After cooling to ~45 °C and dropwise addition of acetic anhydride until massive bubbling occurred, the solution was further cooled to room temperature, and the formulated THCA was extracted with diethyl ether and dried (85% yield). The corresponding 25-chloronorcholane was then obtained by dissolving 0.2 mmol of the formulated THCA in 2.5 ml of dry benzene containing 0.4 mmol of lead tetracetate and 0.4 mmol of LiCl and heating to 85 °C for 3 h. At this time and again at 4 h, 0.2 mmol of LiCl was added. The reaction mixture was then cooled, centrifuged to remove precipitated material, washed with ice-cold 2% NaOH and ice-cold water, dried with MgSO4, and evaporated to dryness to afford 25-chloronorcholane (100%; 1.55 mCi; 77% yield). Finally, the purified nitrile was dissolved in 1 ml of dry dimethyl sulfoxide containing 2.0 mCi of Na[14C]CN (36 μCi, 55 mCi/mmol) and heated for 32 h at 70 °C. After cooling, 1 ml of 2 N NaOH was added, and the solution was heated to 90 °C for 1 h to hydrolyze the formyl group. The product (cyano-3a,7a,12a-trihydroxy[26-14C]norcholesterol) was then extracted with chloroform, dried, and purified by preparative thin-layer chromatography in cyclohexane/ethyl acetate/acetone (21:69:9; 1.55 mCi; 77% yield). This method was used to produce C-acyl-CoA derivatives of bile acids (13).

**Full-length cDNAs encoding hVLCS and hBACS were cloned into the expression vector pcDNA3 (Invitrogen) as previously described (8, 12). Cloning of N-acyl-hBCAS was also described previously (12).**

**Construction of N-acyl-hVLCS**—The full-length open reading frame of hVLCS was amplified by PCR using oligonucleotide primers P0-17 (5’-CCCGTGACCAATGTTTCCGGCCTACTCA-3’, which incorporates a SalI restriction site) and P0-4 (5’-TGTGCGGGCGCTCCTGCAACAGCTGATTGGC-3’, which incorporates a NorI site) with hVLCS in pcDNA3 as template. The product was ligated into the XhoI and NorI sites of N-acyl-pcDNA3, a modified pcDNA3 vector that places the c-myc epitope in-frame at the amino terminus (a gift of Dr. S. Gould). To produce C-acyl-hVLCS, a three fragment ligation was performed. A 1614-bp fragment containing the initiator methionine codon was excised from hVLCS in pcDNA3 using BamHI and BglI. A 550-bp fragment of hVLCS in which the stop codon was replaced by a HindIII restriction site was amplified by PCR using hVLCS in pcDNA3 as template and oligonucleotide primers P0-17 (5’-CCGGTCTTCGACGTTTCGGTCGGCTACTCA-3’, which incorporates a SalI restriction site) and P0-4 (5’-TGTGCGGGCGCTCCTGCAACAGCTGATTGGC-3’, which incorporates a NorI site) with hVLCS in pcDNA3 as template. The product was ligated into the XhoI and NorI sites of N-acyl-pcDNA3, a modified pcDNA3 vector that places the c-myc epitope in-frame at the amino terminus (a gift of Dr. S. Gould). Expressed sequence tag (EST) clones representing the murine homolog of hVLCS were identified by homology probing of the EST data base using rat VLCS cDNA sequence (13) as the query. An EST clone (GenBank™ accession number AA038113) containing the C-terminal 921 base pairs of the open reading frame plus 8 bp of the 3′-untranslated region was obtained from a mouse EST database. The EST clone was inserted into the mammalian cloning vector pcDNA3 using EcoRI and NorI. The 5′-untranslated region of rat VLCS (13) was used to design a forward PCR primer (5′-CCGGGTAATCCGCTTACCC-3′) with an engineered KpnI site and to amplify the 5′-end of mouse VLCS from liver total cDNA with a reverse primer (7′-GACCCCAGACG-5′). The 5′- and 3′-PCR products encompassed a second KpnI site near the 3′-end. Both KpnI sites in the PCR fragment were used to clone the PCR fragment into a KpnI site near the 5′-end of EST AA038113 in pcDNA3, and the clone was re-sequenced. The full-length open reading frame of mouse VLCS was deposited in the GenBank™/EBI Data Bank (accession number AF033031).

**VLCS and Bile Acid Metabolism**—The method of Giger et al. (16) was used, with modifications. Plasmids containing full-length cDNA encoding the murine homologs of VLCS and BACS in pcDNA3 were linearized by cutting at a restriction site upstream of the initiator methionine codon (for VLCS) or at oligonucleotide primer I and a second fragment of the restriction site was used. The stop codon was replaced by a HindIII restriction site was amplified by PCR using hBACS in pcDNA3 as template with oligonucleotide primers P0-19 (5’-CCGGGTAATCCGCTTACCC-3′, forward) and P0-18 (5′-TATAGGGTGAAGTCCTACGAGTC-3′, reverse); this fragment was cut with HindIII and HindIII, yielding a 270-bp fragment. These two pieces were ligated into the KpnI and HindIII sites of pcDNA3.1-Myec-Hisα, forming C-acyl-hBACS.

**In Situ Hybridization**—The method of Giger et al. (16) was used, with modifications. Plasmids containing full-length cDNA encoding the murine homologs of VLCS and BACS in pcDNA3 were linearized by cutting at a restriction site upstream of the initiator methionine codon for VLCS by either SpeI and enzyme. In the experiments shown in Figs. 1A and 3B, the gradient was adjusted to provide a 15-min incubation of bile acid cDNA with a single isoflourescent derivative for elution from the HPLC column (25–55% solvent B over 30 min), which deviated from the previously described method (25–50% solvent B over 25 min) (7). The unlabeled THCA used in these experiments also contained Δ24-THCA (8%, w/w). In the HPLC tracing shown in Fig. 1D, peak 3 was tentatively identified as the CoA derivative of Δ24-THCA. The retention time of this product was identical to that of Δ24-THCA-CoA produced from the incubation of 24-hydroxy-THCA (varanoyl-CoA) with fibroblasts from patients with mutations in the 3-hydroxyacyl-CoA dehydrogenase domain of peroxisomal D-bifunctional protein. Tracings from patients with mutations in the enoyl hydratase portion of the gene had severe reductions in this product, as described previously (15). For inhibition experiments (see Fig. 3B), (25S)-THCA-CoA was quantitated using a Hewlett-Packard 3392A integrator. Activation of [1-14C]palmitic acid by transfected COS-1 cells was measured as described (7), with the following exceptions. The poorly soluble sodium salt of lithocholate was dried on the bottom of reaction tubes and was solubilized with 50 μl of β-cyclodextrin (10 mg/ml) in 10 ml Tris (pH 8.0) by brief sonication prior to addition of other reaction components and enzyme. In the experiments shown in Figs. 1C and 3B, the gradient was adjusted to provide a 15-min incubation of bile acid cDNA with a single isoflourescent derivative for elution from the HPLC column (25–55% solvent B over 30 min), which deviated from the previously described method (25–50% solvent B over 25 min) (7). The unlabeled THCA used in these experiments also contained Δ24-THCA (8%, w/w). In the HPLC tracing shown in Fig. 1D, peak 3 was tentatively identified as the CoA derivative of Δ24-THCA. The retention time of this product was identical to that of Δ24-THCA-CoA produced from the incubation of 24-hydroxy-THCA (varanoyl-CoA) with fibroblasts from patients with mutations in the 3-hydroxyacyl-CoA dehydrogenase domain of peroxisomal D-bifunctional protein. Tracings from patients with mutations in the enoyl hydratase portion of the gene had severe reductions in this product, as described previously (15). For inhibition experiments (see Fig. 3B), (25S)-THCA-CoA was quantitated using a Hewlett-Packard 3392A integrator. Activation of [1-14C]palmitic acid by transfected COS-1 cells was measured as previously described (8).
When chenodeoxycholate was the substrate, a product with a retention time of 20.3 min was detected in cells expressing hBACS (Fig. 1A). No product was detected in cells transfected with hVLCS, hVLCS-H1, rat liver long-chain acyl-CoA synthetase-1 (rACS1), or the vector (pcDNA3) alone (Fig. 1A). We had previously reported that no product is formed in the absence of ATP, CoA, or the bile acid substrate (7) (data not shown). To substantiate the results of the HPLC-based assay, we incubated COS-1 cells expressing hBACS and related proteins with radiolabeled chenodeoxycholate (Fig. 2A). Again, only cells expressing hBACS were capable of catalyzing chenodeoxycholate activation. Thus, we conclude that chenodeoxycholate is also a substrate for hBACS.

Similar results were obtained using the HPLC-based assay when the secondary bile acids deoxycholate (Fig. 1B) and lithocholate (Fig. 1C) were substrates for activation by hBACS. The putative products deoxycholoyl-CoA and lithocholoyl-CoA eluted at 21.7 and 19.7 min, respectively. No product was detected with either substrate in cells expressing hVLCS, hVLCS-H1, or rACS1, consistent with the hypothesis that the liver-specific hBACS is a bile acyl-CoA synthetase capable of activating both primary and secondary bile acids.

Both hVLCS and hBACS Activate the Bile Acid Precursor THCA—Based on the above observations, we predicted that the C27 precursor of cholic acid (THCA) would be activated to its CoA derivative by hBACS, but not by the other hVLCSs or rACS1. When THCA was incubated with ATP, MgCl2, CoA, and COS-1 cells expressing hBACS, three product peaks were seen on the HPLC tracing (Fig. 1D). The substrate used is a racemic mixture of (25S)- and (25R)-THCA stereoisomers. It was previously shown that the physiologic isomer ((25S)-THCA) elutes slightly earlier than the non-physiologic isomer ((25R)-THCA).
in a similar HPLC system (19). Thus, we have provisionally designated peak 1 (23.4 min) as (25S)-THCA and peak 2 (23.7 min) as (25R)-THCA. Peak 3 had a retention time (22.3 min) identical to that of 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oyl-CoA (see "Experimental Procedures") (data not shown). This compound is the product formed by the action of peroxisomal branched-chain acyl-CoA oxidase on (25S)-THCA and accumulates when fibroblasts from children with mutations in peroxisomal branched-chain acyl-CoA oxidase are incubated with 24-OH-THCA (15).

Similar to the situation with primary and secondary bile acids, no product was detected when THCA was incubated with COS-1 cells expressing hVLCS, rACS1, or the vector alone (Fig. 1D). In contrast, cells expressing hVLCS (which were not capable of activating the bile acids) yielded the same three product peaks as did cells expressing hBACS (Fig. 1D). This observation suggests that both hVLCS and hBACS are capable of activating THCA for its subsequent conversion to cholate.

The ability of hVLCS and hBACS to activate THCA was confirmed by radiochemical assay using [26-14C]THCA as substrate. COS-1 cells expressing either hVLCS or hBACS exhibited THCA-CoA synthetase activity (Fig. 2B). COS-1 cells transfected with the vector (pcDNA3) alone, hVLCS-H1, or rACS1 were unable to activate [26-14C]THCA (Fig. 2B).

**THCA Inhibits hBACS-catalyzed Activation of Cholate**—To elucidate the role(s) of hVLCS and hBACS in bile acid metabolism, we first investigated the ability of the biosynthetic precursor of cholate (THCA) to inhibit cholate activation. We incubated COS-1 cells expressing hBACS with radiolabeled cholate in the presence of increasing concentrations of THCA. A concentration of THCA (4 μM) that was 20% of the cholate concentration (20 μM) reduced cholate activation by nearly half (Fig. 3A). When THCA was present at the same (20 μM) and at double (40 μM) the cholate concentration, we observed 72 and 82% inhibition, respectively (Fig. 3A).

**Cholate Inhibits Activation of THCA by hBACS, but Not by hVLCS**—Because cholate is a substrate for hBACS, but not for hVLCS, we next examined the effect of unlabeled cholate on THCA activation by these two enzymes. When COS-1 cells expressing hBACS were incubated with 20 μM [1-14C]cholate and the indicated concentration of unlabeled THCA. The production of labeled choloyl-CoA was quantitated as described under "Experimental Procedures." The results are the means ± S.E. from three separate transfection experiments. Because of the limited availability of [26-14C]THCA, the results in B are the mean of two separate transfections.

**Kinetic Studies of Cholate and Palmitate Activation**—Kinetic studies were performed to establish further that hVLCS does not activate bile acids and that hBACS does not activate long-chain fatty acids. COS-1 cells expressing hBACS exhibited normal Michaelis-Menten kinetics when cholate was the substrate (data not shown). Under these conditions, the $K_m$ of hBACS for cholate was 2.8 μM, and the $V_{max}$ was 3.8 nmol/20 min/mg of COS cell protein, as determined by nonlinear regression analysis. Similar experiments were performed using the substrate palmitic acid (C16:0), a
long-chain fatty acid that is the preferred substrate for hVLCS (8). Kinetic analysis is complicated by the fact that untransfected and vector-transfected COS-1 cells contain several endogenous acyl-CoA synthetases that can activate C16:0 (see Fig. 4B); thus, endogenous activity must be subtracted from that of hBACS-transfected cells. At each of five C16:0 concentrations tested (range of 2.5–40 μM), there was no statistically significant increase in C16:0 activation by hBACS-transfected cells compared with pcDNA3-transfected cells (p > 0.1, n = 3; data not shown). It is therefore unlikely that C16:0 is activated to a significant degree by hBACS in vivo.

The kinetics of C16:0 and cholate activation by COS-1 cells expressing hVLCS were also examined. When C16:0 was the substrate, both pcDNA3- and hVLCS-transfected COS-1 cells exhibited apparently normal Michaelis-Menten kinetics (see Fig. 4B). Nonlinear regression analysis revealed a Km of 48 μM and a Vmax of 167 nmol/20 min/mg of COS cell protein for vector-transfected cells and a Km of 32 μM and a Vmax of 175 nmol/20 min/mg of COS cell protein for cells expressing hVLCS. The contribution of hVLCS to the overall C16:0-CoA synthetase activity was estimated by subtraction (Fig. 4B), and a Km of 12 μM and a Vmax of 28 nmol/20 min/mg of COS cell protein were calculated. It must be emphasized that these derived values are, at best, crude estimates. In our previous study (7), we found that neither vector-transfected nor hVLCS-transfected COS-1 cells activate 20 μM cholate to its CoA derivative. We now report that no detectable activation was observed at any of five concentrations of cholate tested (range of 2.5–40 μM) (data not shown).

Hepatic Organization of VLCS- and BACS-expressing Cells—We hypothesize that the primary metabolic role of hBACS is bile acid activation. On the other hand, although hVLCS may play a role in bile acid synthesis from THCA, this enzyme can activate a wide variety of substrates, including long-, very long-, and branched-chain fatty acids (7, 8). If these two synthetases have different functions, the intracellular localization of cells expressing them might differ. We used in situ hybridization to address this question in mouse liver. Using an
antisense RNA probe synthesized from cDNA encoding the murine ortholog of hVLCS, we found that hepatocytes uniformly expressed this enzyme (Fig. 5, D and E). In contrast, hepatocytes expressing the murine homolog of hBACS mainly had a perportal distribution (Fig. 5, A and B). These observations are consistent with the differing proposed roles of the two acyl-CoA synthetases in bile acid and fatty acid metabolism.

Topographic Orientation of hVLCS and hBACS Expressed in COS-1 Cells—We previously reported that although hBACS is primarily a microsomal enzyme, hVLCS is localized to both peroxisomes and the endoplasmic reticulum (8, 20). Other laboratories have reported that activation of both bile acids (17, 18, 21) and the bile acid precursors DHCA and THCA (22) occurs in microsomes. The fact that hBACS activates both bile acids and THCA is consistent with these reports. Our observation that hVLCS, with its dual subcellular location, can activate THCA, but not bile acids, raised questions about the precise role(s) of hVLCS and hBACS in bile acid synthesis.

An initial approach to answer this question was to examine the topographic orientation of hVLCS and hBACS in the endoplasmic reticulum membrane. For these studies, epitope tags were placed at either end of the expressed proteins. Full-length hVLCS cDNA clones incorporating an in-frame myc epitope at the N and C termini were constructed as outlined under “Experimental Procedures.” Similar myc-tagged constructs of hBACS were prepared. COS-1 cells expressing these cDNAs were examined by indirect immunofluorescence using anti-myc antibody to determine the orientation of the expressed protein. We previously reported that when COS-1 cells expressing hVLCS are permeabilized with Triton X-100, the protein is detected primarily in the endoplasmic reticulum, whereas the endogenous protein is also detected in peroxisomes (8, 20). COS-1 cells transfected with either N-myc-hVLCS (Fig. 6A) or C-myc-hVLCS (Fig. 6D) also had a staining pattern consistent with expression in the endoplasmic reticulum. The location was confirmed by colocalization of immunofluorescence of hVLCS with that of the endoplasmic reticulum luminal resident protein calreticulin (Fig. 6, B and F). Also as reported previously (12), an endoplasmic reticulum N-myc-hBACS staining pattern was observed in Triton X-100-permeabilized COS-1 cells expressing this protein (Fig. 6F). Cells expressing C-myc-hBACS had an identical immunostaining pattern (Fig. 6M).

Permeabilization of cells with digitonin rather than Triton X-100 yielded a somewhat different result. The C-myc-hVLCS protein was detected in digitonin-permeabilized COS-1 cells transfected with its cDNA (Fig. 6G), but N-myc-hVLCS was not visualized (Fig. 6C). Under the conditions utilized, the luminal protein calreticulin could not be detected (Fig. 6, D and H). These data indicate that although the C terminus of hVLCS faces the cytoplasm, the N terminus faces the lumen of the endoplasmic reticulum. We previously reported that the C terminus of endogenous hVLCS is oriented toward the peroxisomal matrix and not the cytoplasm (20). In contrast, both the N-myc-hBACS (Fig. 6K) and C-myc-hBACS (Fig. 6O) proteins could be detected in digitonin-permeabilized cells (compare with calreticulin) (Fig. 6, L and P). These data suggest that hBACS may preferentially act on substrates present in the cytoplasm.

DISCUSSION

The synthesis of bile acids from cholesterol, conjugation with amino acids, secretion into the bile and subsequently into the
intestinal tract, reabsorption from the intestine, and reutilization require the integrated activity of multiple enzymes, organelles, and tissues. At two points in the hepatic phase of bile acid metabolism, activation of the carboxylic acid side chain of the steroid to a CoA derivative is required. First, in the pathway of de novo synthesis from cholesterol, the C27 precursors of cholate and chenodeoxycholate (THCA and DHCA, respectively) must be converted to THCA-CoA and DHCA-CoA for cleavage of three carbons from the side chain to occur. Second, to conjugate the C24 bile acids cholate and chenodeoxycholate to glycine or taurine, their carboxylic acid side chains must first be activated to the corresponding CoA derivatives. The studies reported here begin to address the nature of the acyl-CoA synthetases that are involved in these two aspects of bile acid metabolism.

Based on the biochemical and morphological data presented here and our previous findings (7), we have developed a working hypothesis regarding the role(s) of hVLCS and hBACS in bile acid metabolism (Fig. 7). Conversion of cholesterol to THCA and DHCA requires several enzymatic steps that take place in the endoplasmic reticulum, cytosol, and mitochondria (1, 2). Activation of THCA and DHCA, precursors of cholate and chenodeoxycholate, respectively, could be catalyzed by either hVLCS or hBACS in the endoplasmic reticulum or by hVLCS in peroxisomes. Once inside peroxisomes, the side chains of THCA-CoA and DHCA-CoA are shortened by three carbons via β-oxidation, yielding choloyl-CoA and chenodeoxycholoyl-CoA, respectively. The peroxisomal enzyme bile acyl-CoA:amino-acid N-acyltransferase then catalyzes the formation of the glycine and taurine conjugates of choloyl-CoA and chenodeoxycholoyl-CoA, respectively. Exiting the peroxisome, the conjugated bile acids are secreted into bile canaliculi and eventually reach the small intestine. Although a large proportion of the conjugated bile acids remain intact, a small quantity undergoes conversion in the bowel. Intestinal flora catalyze both deconjugation and dehydroxylation reactions (1, 2). 7α-Dehydroxylation of cholate and chenodeoxycholate yields the secondary bile acids deoxycholate and lithocholate, respectively. Intestinal metabolism thus yields a mixture of primary and secondary bile acids that includes both conjugated and free acids. Reabsorption of free bile acids occurs by passive diffusion throughout the small bowel (1, 24). When these free acids reach the liver via the portal circulation, they must be reactivated and then conjugated before they are returned to the bile. hBACS is uniquely positioned in the endoplasmic reticulum membrane to catalyze the reactivation reaction. Bile acyl-CoA:amino-acid N-acyltransferase is found in the cytoplasm as well as the peroxisome (17, 25). Following re-conjugation in the cytoplasm, primary and secondary bile acids can be secreted into the bile for reutilization. This hypothesis is consistent with that recently proposed by Solaas et al. (25), who investigated bile acyl-CoA:amino-acid N-acyltransferase in human liver.

Using subcellular fractions from rat liver, other investigators have shown that activation of THCA to its CoA derivative occurs primarily in microsomes (22, 25). Our data indicate that both hVLCS and hBACS are capable of activating THCA and that both enzymes are found in the endoplasmic reticulum. Because hVLCS is also found in peroxisomes (8, 20), it is not clear why Schepers et al. (22) and Solaas et al. (25) did not detect significant THCA activation in peroxisome-enriched liver fractions. Activation of THCA in peroxisomes rather than in microsomes is a more attractive hypothesis because CoA derivatives generally do not
cross biological membranes. hVLCS is oriented in the peroxi-
smal membrane facing the matrix (20, 26), and activation of
THCA would use intraperoxisomal CoA-SH. Amidation of the
β-oxidation product, choloyl-CoA, by peroxisomal bile acyl-CoA:
amino-acid N-acyltransferase would regenerate free CoA-SH in-
side the organelle.

Alternatively, if activation of THCA takes place solely in the
endoplasmic reticulum, the CoA-SH balance must somehow be
maintained. A possible mechanism for maintaining a constant
intraperoxisomal CoA-SH pool is one in which THCA-CoA en-
tering the organelle is coupled to CoA-SH exiting. However, it
is not clear at this time how either THCA or THCA-CoA enters
peroxisomes. Furthermore, it remains to be determined
whether THCA activation reaction is catalyzed primarily by
VLCS or by BACS, if this process does not occur in peroxi-
somes. Data reported by Schepers et al. (22) on pH dependence
and Triton X-100 sensitivity indicate that in rat liver micros-
omes, the enzyme catalyzing THCA activation is distinct from
that catalyzing cholate activation. This suggests that VLCS
and not BACS might be the primary enzyme that activates
THCA. On the other hand, Wheeler et al. (27) purified a bile
acid:CoA ligase from rat liver (most likely rat BACS) that
activated both chenodeoxycholate and THCA. We plan to ad-
dress this issue in future studies by investigating THCA acti-
vation in subcellular fractions prepared from livers of mice in
which the VLCS gene has been disrupted.2

The data presented here suggest that hVLCS activates
THCA more robustly than does hBACS and is not sensitive to
inhibition by cholate. These results are consistent with the
notion that hVLCS has a diverse role in metabolism, activating
both fatty acids and THCA. In contrast, hBACS showed a
greater preference for bile acids and their precursors as sub-
strates, suggesting a more limited role for this enzyme (7, 12).
THCA inhibition of cholyoyl-CoA synthase was stronger than
cholate inhibition of THCA-CoA synthase in hBACS-trans-
celled cells. These data are consistent with those reported by
Schepers et al. (22) for rat liver microsomes.

The peroxisomal pathway for chain shortening of the C\textsubscript{27}
compounds THCA-CoA and DHCA-CoA to C\textsubscript{24} bile acids by
β-oxidation is now well established (3). In humans, the first
step is catalyzed by branched-chain acyl-CoA oxidase, the same
enzyme that oxidizes α-methyl-branched fatty acids such as
pristanic acid (28). In rodents, distinct enzymes carry out these
two processes (29). D-bifunctional protein catalyzes the second
(enoic-CoA hydratase) and third (hydroxyacyl-CoA dehydro-
genase) activities (30). The thiolase associated with the 58-kDa
protein sterol carrier protein X is thought to carry out the final
reaction, thiolic cleavage and release of propionyl-CoA (31). It
is noteworthy that the C\textsubscript{24} products of the thiolase reaction are
the CoA derivatives of cholic acid andchenodeoxycholic acid
and not the free acids. Furthermore, bile acyl-CoAminooxid-
N-acyltransferase, the conjugating enzyme, contains a peroxi-
somal targeting signal and can be detected in this organelle
(23). Thus, peroxisomes possess the complete enzymatic
machinery required to convert THCA and DHCA into conjugated
bile acids.

In situ hybridization studies are consistent with the pro-
posed roles of VLCS and BACS in bile acid and fatty acid
metabolism. Hepatic lipogenic enzymes appear to have a more
perivenous distribution (32, 33), whereas the rate-limiting step
in bile acid synthesis, cholesterol 7α-hydroxylation, is found
mainly in periportal hepatocytes (34). Expression of hBACS
was also found to have a more portal distribution. The
enterohepatic circulation returns bile acids to the liver via the
portal vein; thus, hBACS is well positioned to efficiently “scav-
gen” these bile acids, reactivating them for recycling. In con-
trast, hVLCS, which robustly activates fatty acids as well as
THCA, had a more uniform distribution throughout the liver
acinus.

The topographic orientation of hBACS, in which both the C
and N termini face the cytoplasm, also makes it well suited for
its proposed role in reactivation of bile acids returning to the
hepatocytes via the enterohepatic circulation. Analysis of the
hBACS amino acid sequence by the PHDhtm algorithm re-
vealed that the protein contains no unequivocal transmem-
brane-spanning domains (35). The orientation of hVLCS in the
endoplasmic reticulum membrane, with the N terminus facing
the lumen and the C terminus facing the cytoplasm, is consist-
ent with the model proposed by Smith et al. (26). These authors
suggested that VLCS is an integral membrane protein in mi-
icrosomes. Schepers et al. (22) were unable to release THCA-
CoA synthetase activity from microsomes using high salt alc-
one, consistent with the notion that VLCS and not BACS is primar-
ily responsible for this synthetase activity.

In summary, in this report, we have described the ability of
two members of the VLCS/fatty acid transport protein family
to activate bile acids and their precursors. Based on these data,
we presented a working hypothesis regarding the roles of these
enzymes in bile acid metabolism. Solaas et al. (25) recently
hypothesized that the peroxisomal conversion of THCA to
choloyl-CoA is the rate-limiting step in production of conju-
gated bile acids via de novo synthesis. They further proposed
that the rate-limiting step in the reutilization of bile acids
returning to the liver via the enterohepatic circulation is the
reactivation step (25). Future studies will enable us to test
these hypotheses.

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REFERENCES
1. Setchell, K. D. R., and O’Connell, N. C. (2001) in Liver Disease in Children
(Suchy, F. J., Sokol, R. J., and Balisteri, W. F., eds) 2nd Ed., pp. 701–733,
Lippincott Williams & Wilkins, Philadelphia
2. Vlahcevic, Z. R., Pandak, W. M., and Stravitz, R. T. (1999) Gastroenterol.
Clin. North Am. 28, 1–25
3. Wanders, R. J. A., Barth, P. C., and Heymans, H. S. A. (2001) in The Metabolic
& Molecular Bases of Inherited Disease (Online, C. R., Beaudet, A. L., Sly,
W. S., and Valle, D., eds) 8th Ed., pp. 3219–3256, McGraw-Hill Book Co.,
New York
4. Kase, F., Bjorkhem, I., and Pedersen, J. I. (1983) J. Lipid Res. 24, 1560–1567
5. Hofmann, A. F. (1994) in The Liver: Biology and Pathobiology (Arias, A. M.,
Beyer, J. L., Fausto, N., Jakoby, W. B., Schachter, D. A., and Shafritz, D. A.,
eds) 3rd Ed., pp. 677–718, Raven Press, New York
6. Carey, M. C., and Duane, W. C. (1994) in The Liver: Biology and Pathobiology
(Arias, A. M., Boyer, J. L., Fausto, N., Jakoby, W. B., Schachter, D. A., and
Shafritz, D. A., eds) 3rd Ed., pp. 719–767, Raven Press, New York
7. Steinberg, S. J., Wang, S. J., Kim, D. G., Mihalik, S. J., and Watkins, P. A.
(2000) J. Biol. Chem. 275, 15605–15608
8. Steinberg, S. J., Wang, S. J., Kim, D. G., Mihalik, S. J., and Watkins, P. A.
(1999) Biochem. Biophys. Res. Commun. 257, 615–621
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol.
Chem. 193, 265–275
10. Xu, R. F., and Cuebas, D. A. (1996) Biochem. Biophys. Res. Commun. 221,
271–278
11. Tseng, K. Y., and Klein, P. D. (1977) J. Lipid Res. 18, 400–403
12. Steinberg, S. J., Wang, S. J., McGuinness, M. C., and Watkins, P. A. (1999)
Mol. Genet. Metab. 68, 32–42
13. Uchiyama, A., Aoyama, T., Kamijo, K., Uchida, M., Otsuki, M., and
Hashimoto, T. (1996) Am. J. Hum. Genet. 58, 252–265
14. Watkins, P. A., Gould, S. J., Smith, M. A., Braiterman, L. T., Wei, H.-M., Kok
F., Moser, A. B., Moser, H. W., and Smith, K. D. (1995) Am. J. Hum. Genet.
47, 292–301
15. van Groenen, E. G., van Berkel, E., IJlst, L., Vreken, P., de Klerk, J. B.,
Adamski, J., Lemonde, H., Clayton, P. T., Cuebas, D. A., and Wanders, R. J.
(1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2128–2133
16. Giger, R. J., Wolfer, D. P., De Wit, G. M., and Verhaagen, J. (1996) J. Comp.
Neurol. 375, 378–392
17. Killenberg, P. G. (1978) J. Lipid Res. 19, 24–31
18. Polokoff, M. A., and Bell, R. M. (1977) J. Biol. Chem. 252, 1167–1171

2 A. K. Heinzer, P. A. Watkins, and K. D. Smith, unpublished data.
19. Kurosawa, T., Sato, M., Inoue, K., Yoshimura, T., Tohma, M., Jiang, L. L., and Hashimoto, T. (1998) Anal. Chim. Acta 365, 249–257
20. Steinberg, S. J., Kemp, S., Braiterman, L. T., and Watkins, P. A. (1999) Ann. Neurol. 46, 409–412
21. Lim, W. C., and Jordan, T. W. (1981) Biochem. J. 197, 611–618
22. Schepers, L., Casteels, M., Verheyden, K., Purmentier, G., Asselberghs, S., Eyssen, H. J., and Mannaerts, G. P. (1989) Biochem. J. 257, 221–229
23. Kase, B. F., and Bjorkhem, I. (1989) J. Biol. Chem. 264, 9220–9223
24. Bahar, R. J., and Stolz, A. (1999) Gastroenterol. Clin. North Am. 28, 27–58
25. Solaas, K., Ulvestad, A., Soreide, O., and Kase, B. F. (2000) J. Lipid Res. 41, 1154–1162
26. Smith, B. T., Sengupta, T. K., and Singh, I. (2000) Exp. Cell Res. 254, 309–320
27. Wheeler, J. B., Shaw, D. R., and Barnes, S. (1997) Arch. Biochem. Biophys. 348, 15–24
28. Vanhove, G. F., Van Veldhoven, P. P., Fransen, M., Denis, S., Eyssen, H. J., Wanders, R. J. A., and Mannaerts, G. P. (1993) J. Biol. Chem. 268, 10335–10344
29. Van Veldhoven, P. P., Vanhove, G., Asselberghs, S., Eyssen, H. J., and Mannaerts, G. P. (1992) J. Biol. Chem. 267, 20065–20074
30. Dieuaide-Noubhani, M., Novikov, D., Baumgart, E., Vanhooren, J. C., Fransen, M., Goethals, M., Vandekerckhove, J., Van Veldhoven, P. P., and Mannaerts, G. P. (1996) Eur. J. Biochem. 240, 669–668
31. Antonenkov, V. D., Van Veldhoven, P. P., Waeltens, E., and Mannaerts, G. P. (1997) J. Biol. Chem. 272, 26023–26031
32. Katz, N., Thiele, J., and Giffhorn-Katz, S. (1989) Eur. J. Biochem. 180, 185–189
33. Katz, N. R., Fischer, W., and Giffhorn, S. (1983) Eur. J. Biochem. 135, 103–107
34. Twisk, J., Hoekman, M. P., Mager, W. H., Moorman, A. F., de Boer, P. A., Schols, L., Princen, H. M., and Gebhardt, R. (1995) J. Clin. Invest. 95, 1235–1243
35. Rost, B., Casadio, R., Fariselli, P., and Sander, C. (1995) Protein Sci. 4, 521–533
