Molecular cloning and expression of rat liver bile acid CoA ligase

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Abstract **Bile acid CoA ligase (BAL) is responsible for catalyzing the first step in the conjugation of bile acids with amino acids. Sequencing of putative rat liver BAL cDNAs identified a cDNA (rBAL-1) possessing a 51 nucleotide 5′-untranslated region, an open reading frame of 2,070 bases encoding a 690 aa protein with a molecular mass of 75,960 Da, and a 138 nucleotide 3′-nontranslated region followed by a poly(A) tail. Identity of the cDNA was established by: *1*) the rBAL-1 open reading frame encoded peptides obtained by chemical sequencing of the purified rBAL protein; *2*) expressed rBAL-1 protein comigrated with purified rBAL during SDS-polyacrylamide gel electrophoresis; and *3*) rBAL-1 expressed in insect Sf9 cells had enzymatic properties that were comparable to the enzyme isolated from rat liver. Evidence for a relationship between fatty acid and bile acid metabolism is suggested by specific inhibition of rBAL-1 by cis-unsaturated fatty acids and its high homology to a human very long chain fatty acid CoA ligase. In summary, these results indicate that the cDNA for rat liver BAL has been isolated and expression of the rBAL cDNA in insect Sf9 cells results in a catalytically active enzyme capable of utilizing several different bile acids as substrates.**—Falany, C. N., X. Xie, J. B. Wheeler, J. Wang, M. Smith, D. He, and S. Barnes. **Molecular cloning and expression of rat liver bile acid CoA ligase.** ***J. Lipid Res.*** **2002.** 43: **2062–2071.**

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Bile acids are one of a group of organic acids that are converted to *N*-acyl amino acid conjugates. They are the principal metabolites of cholesterol and have important detergent properties in the hepatobiliary system and in the lumen of the upper small intestine (1). The presence of the amino acid moiety lowers the pKa of the bile acid, thus preventing precipitation at the acid pH of the upper gut (2). Conjugation of bile acids with the amino acids glycine and taurine is a two-step process involving the successive action of the enzymes, bile acid CoA ligase (BAL), and bile acid CoA:amino acid *N*-acyltransferase (BAT). Our laboratories have previously purified BAT activity from human liver (3), and cloned a cDNA encoding hBAT from a human liver cDNA library (4). hBAT expressed in both bacterial and COS cells was demonstrated to utilize both glycine and taurine as substrates (4).

Although BAL activity was identified and enzymatically characterized over 40 years ago (5), purification of membrane-bound BAL activity has been a major challenge. Partial purification and characterization of BAL activity from guinea pig and porcine microsomes has been reported (6); however, the multiplicity and properties of the BAL proteins were not determined. Recently, our laboratory has described the purification of BAL activity from rat liver (7). Monoclonal anti-rBAL antibodies were generated and used to isolate rBAL so that partial amino acid sequence information could be obtained by chemical sequencing methods. The sequence of several peptides was utilized to design degenerate oligonucleotide probes for the cloning of rBAL cDNAs from a rat liver λZAP II cDNA library. The full-length rBAL-1 cDNA was expressed in insect Sf9 cells using a baculovirus expression vector. Verification that the expressed protein was rBAL involved identification of rBAL peptide sequences in the translation of the cDNA, characterization of the enzymatic activity of expressed rBAL, and identification of the formation of bile acid CoA esters using HPLC and MALDI-TOF mass spectrometry. Sequence analysis and substrate inhibition studies indicate that rBAL is a member of the fatty acid CoA synthetase/fatty acid transport protein (FATP) gene family (8).

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Abbreviations: BAL, bile acid CoA ligase; BAT, bile acid CoA:amino acid *N*-acyltransferase; CA, cholic acid; CDCA, chenodeoxycholic acid; CoA, coenzyme A; DCA, deoxycholic acid; FATP, fatty acid transport protein; LCA, lithocholic acid; THCA, trihydroxycoprostanoic acid.

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MATERIALS AND METHODS

Materials

TRIzol reagent was purchased from Gibco BRL (Rockville, MD). Original TA Cloning and PCR optimizer kits were obtained from Invitrogen (Carlsbad, CA). The Wizard Plus Miniprep DNA purification system was from Promega (Madison, WI). The TaqStart Antibody was purchased from Clontech (Palo Alto, CA). Rat liver λZAP II cDNA library, the XL-1 MRF′ cells, and SOLR cells were from Stratagene (La Jolla, CA). STAT-60 RNA isolation reagent was obtained from TEL-TEST (Friendswood, TX). First Strand Synthesis, Oligolabeling and ECL Plus western blot detection kits were purchased from Pharmacia Biotech (Arlington Heights, IL). QIAEX II gel purification kit was from Qiagen (Valencia, CA). Unlabeled chenodeoxycholic acid (CDCA), cholic acid (CA), lithocholic acid (LCA), deoxycholic acid (DCA), and coenzyme A (CoA) were from Sigma Chemical Co. (St. Louis, MO). Trihydroxycoprostanoic acid (THCA) and norcholic acid were gifts from Dr. Alan Hofmann (University of California, San Diego). [11,12-^{3}H]CDCA was from NEN/Dupont (Wilmington, DE). [9,10-^{3}H(N)]Oleic acid was from Perkin Elmer Life Sciences, Inc. (Boston, MA). All other reagents were of reagent grade and were from Fisher (Norcross, GA).

Molecular cloning of the 3′-end of rBAL

Portions of the rBAL amino acid sequence were obtained from proteolytic trypsin digestion and CNBr cleavage of affinity-purified rBAL (7). Peptide fragments from digested rBAL were separated by reverse-phase C_{18} HPLC. Chemical sequencing of rBAL peptides by Edman degradation was performed by Commonwealth Biotechnologies (Richmond, VA). The amino acid sequence of one of the isolated tryptic peptides was NH_2-EGFDVGVIADPLYILD-COOH. An ~8 kDa fragment of rBAL generated by a CNBr digestion (9) was resolved by SDS-PAGE, electrophoretically transferred to a PVDF membrane, and stained with Coomassie Blue. The resulting band was cut from the membrane and subjected to peptide sequencing from the PVDF membrane. The amino acid sequence obtained from this peptide was NH_2-AAVKLAPGKT[G,F]DGQKLY-COOH. The amino acids contained within the brackets were indeterminate at that position.

In order to generate a portion of the rBAL cDNA, rat liver total RNA was isolated using a TRIzol (Life Technologies) extraction procedure. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using a First Strand Synthesis kit. A degenerate oligonucleotide primer was designed using the amino acid sequence determined from the CNBr cleaved fragment. This sequence, 5′-GCICCIGGIAARACIKKIGAYGG-3′ [encodes APGKT(G,F)DG], was used in conjunction with oligo dT18 to perform PCR. Five microliters of the first strand synthesis reaction mix was added to each PCR reaction. Denaturation and extension cycles were performed at 94°C (1 min) and 72°C (2 min), respectively. For the first three cycles, the 1 min annealing temperature was 58°C. The annealing temperature was then lowered to 55°C for three cycles, to 53°C for three cycles, to 50°C for three cycles, and to 48°C for 21 cycles. The final extension reaction was carried out for 7 min at 72°C. Two major PCR products (~450 bp and 300 bp) were generated. These cDNA fragments were gel purified and ligated into the plasmid pCR2.1 using a TA cloning kit. The ligated plasmid was subcloned into *Escherichia coli* (INVaF′) and grown on LB media agar plates using kanamycin for antibiotic selection and X-gal for color selection. Plasmid DNA was isolated from several colonies using a Wizard Plus Miniprep DNA purification system. The purified plasmid DNAs were sequenced using an ABI model 377 automated sequencer with M13 and T7 sequencing primers (UAB DNA Sequence Core Facility). The larger PCR product (423 bp) was determined to encode the desired peptide sequences.

Isolation of the full-length rBAL cDNA

The 423 bp DNA fragment was labeled with [^{32}P]dCTP using an Oligolabeling kit (Pharmacia) to a specific activity of 1×10^9 cpm/μg DNA and used as a probe to screen a λZAP II Sprague Dawley rat liver cDNA library under high stringency conditions. Screening of the λZAP II cDNA library was carried out as described previously (10). *E. coli* XL-1 Blue MRF′ cells were infected with aliquots of the λZAP library to give approximately 50,000 pfu per 150 mm plate. The phage were allowed to absorb to the bacteria for 15 min at 37°C and then mixed with prewarmed top agar (0.7%) and poured onto Petri plates containing 1.5% agar in Luria broth with 10 mM $MgCl_2$. The plates were incubated overnight at 37°C to allow plaque formation, then overlaid with nitrocellulose filters (Micron Separations, Inc., Westborough, MA) for 5 min. The orientation of the filters was marked with India ink, and the filters were removed. The filters were soaked in 0.5 M NaOH/1.5 M NaCl for 5 min and neutralized with 0.5 M Tris-HCl (pH 7.0)/1.5 M NaCl for 5 min. The filters were then air dried and exposed to UV light (total 150 mJ) to bind the DNA to the filter. Duplicate filters were prepared from each plate.

Approximately 300,000 independent clones from the λZAP II library were screened. Hybridization of the filters was carried out overnight with ~1×10^6 cpm/ml of the ^{32}P-labeled 423 bp DNA fragment at 65°C in 6× SSC, 0.5% SDS, 5× Denhardt's solution, and 200 μg/ml sheared salmon sperm DNA. The filters were washed twice at 65°C for 20 min using 3× SSC containing 0.5% SDS and 0.2% Na_2HPO_4 followed by two 20 min washes at 65°C with 3× SSC. The filters were then air dried and exposed to autoradiograph film at −70°C with an intensifying screen. Plaques that were positive on replicate filters were purified by dilution and re-screening until a single pfu could be isolated. Following isolation of single pfu, recombinant DNA sequences were recovered in pBluescript plasmids using helper phage as described by Stratagene. Plasmid DNA was isolated using a Wizard DNA isolation kit (Promega).

DNA sequence analysis

Isolated recombinant plasmids were sequenced using the Thermo Sequenase cycle sequencing kit (Amersham, Cleveland, Ohio) using [^{33}P]dideoxynucleotide (ddNTP) terminators. The reaction products were resolved on 8% glycerol tolerant polyacrylamide gels. After electrophoresis, the gels were vacuum dried and exposed overnight to autoradiograph film. Sequences were manually read, then aligned and analyzed using MacVector sequence analysis software.

Northern blot analysis of RNA from different rat tissues

Total RNA was isolated from several tissues of an adult male Sprague Dawley rat using STAT-60 (Tel-Test). Aliquots (5 μg) of total RNA from different tissues (liver, kidney, spleen, heart, lung, brain, small intestine and testis) were mixed with 3 vol of RNA gel loading buffer (Ambion), heated to 65°C for 15 min, and resolved in a 1% agarose-formaldehyde gel (11). The RNA was then transferred to a nitrocellulose membrane and the membrane was treated with UV-light in a Bio-Rad UV chamber.

For use as a probe, the rBAL cDNA was isolated from the pBluescript plasmid by *Eco*RI digestion and purified from an agarose gel. The rBAL-1 cDNA was labeled with [^{32}P]dCTP using an oligolabeling kit (Pharmacia) to a specific activity of ~1×10^9 cpm/μg. Membrane hybridization was carried out in Quickhyb solution (Stratagene) at 68°C for 1 h. The membrane was re-

moved and washed twice in 2× SSC with 0.1% SDS for 15 min at RT, followed by washing twice in 0.1× SSC and 0.1% SDS for 15 min at 60°C. The membrane was air-dried and exposed overnight to autoradiograph film at −70°C with an intensifying screen.

Immunoblot analysis of rBAL

A rabbit polyclonal antibody was raised against a 463 amino acid fragment of rBAL because of problems associated with insertion of full-length rBAL into inclusion bodies when expressed in *E. coli*. The rBAL fragment was generated by subcloning the *Bam*H1-*Kpn*1 restriction fragment obtained from pBS-rBAL-1 into pQE-31 (Qiagen) to generate a protein with an amino terminal histidine tag. The rBAL463 fragment was expressed in *E. Coli* M15 cells and purified by Ni-NTA agarose affinity chromatography. The protein was further purified by SDS-polyacrylamide gel electrophoresis and electroelution using a Bio-Rad Model 422 Electro-Eluter and the identity of the protein verified by tryptic peptide fingerprinting using MALDI-TOF mass spectroscopy (PerSeptive Biosystems, Framingham, MA). Pure rBAL463 protein (130 μg) was mixed with Freund's complete adjuvant and injected subcutaneous at several sites along the back of a female New Zealand white rabbit. The rabbit was subsequently boosted twice at two-week intervals with 130 μg of rBAL463 in Freund's incomplete adjuvant. Two weeks later the animal was bled and serum tested for antibody specificity by immunoblot analysis (12).

For immunoblot analysis of rBAL expression in rat tissues, microsomes were prepared from tissues of a young male Sprague Dawley rat and aliquots (80 μg) of the microsomal proteins were resolved by SDS-polyacrylamide gel electrophoresis. The resolved proteins were electrotransferred to a nitrocellulose membrane and the membrane was blocked with 5% non-fat milk. The membrane was then incubated with rabbit polyclonal anti-rBAL antiserum at a dilution of 1:4,000 for 1 h. Goat anti-rabbit IgG horseradish peroxidase conjugate was used as the secondary antibody and visualization of bound proteins was carried out using the Supersignal West Pico System (Pierce).

Expression of rBAL in Sf9 insect cells

Enzymatically active rBAL was expressed in Sf9 insect cells using the pFastBac1 baculovirus expression system (Invitrogen, Carlsbad, CA). The rBAL-1 cDNA was isolated from pBluescript by *Eco*RI and *Spe*I digestion and ligated into the *Eco*RI and *Spe*I sites in the pFastBac1 vector to generate native enzyme or the pFastBacHTb vector to express the protein with a 6x His-tag on the amino end of the protein. Both the pFastBac-rBAL plasmids were transformed into competent *E. coli* DH5α cells and the plasmids isolated with a Wizard plasmid purification kit (Promega). The recombinant pFastBac-rBAL plasmids were then transformed into DH10Bac cells for transposition of the rBAL sequence into the bacmid. Transformed cells were selected on LB agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentimycin, 10 μg/ml tetracycline, 40 μg/ml IPTG, and 100 μg/ml Bluegal. White colonies containing recombinant bacmid DNA were selected for isolation of the large (>23 kb) bacmid DNA as described by Invitrogen.

To isolate the baculovirus containing rBAL, Sf9 cells growing in mid-log phase were seeded at a concentration of 9×10^5 cells/35-mm well in a 6-well plate in 2 ml of Sf-900 II SFM containing penicillin (50 U/ml) and streptomycin (50 μg/ml) and allowed to attach at 27°C for 1 h. For transfection, bacmid DNA, diluted in 100 μl Sf-900 II SFM without antibiotics, was gently mixed with CellFECTIN reagent diluted in 100 μl Sf-900 II SFM with antibiotics and incubated for 45 min at room temperature. The Sf9 cells were washed once with 2 ml Sf-900 II SFM without antibiotics and overlaid with the DNA/CellFECTIN solution diluted with 800 μl Sf-900 II SFM. The cells were incubated at 27°C for 5 h, then the DNA/CellFECTIN solution was removed and replaced with fresh Sf-900 II SFM containing antibiotics. The cells were incubated at 27°C for 72 h and the baculovirus-containing supernatant fraction was removed and centrifuged at 500 *g* for 5 min to remove cells and debris.

To prepare rBAL-containing microsomes, Sf9 cells in mid-log phase in liquid culture were infected with the recombinant virus and incubated at 27°C for 72 h. The Sf9 cells were then harvested by centrifugation at 500 *g* for 5 min. Cell pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4), homogenized in a glass-Teflon homogenizer, and centrifuged at 10,000 *g* for 20 min at 4°C. The supernatant fraction was recovered and centrifuged at 100,000 *g* at 4°C for 1 h. The microsomal pellet was resuspended in 0.1 M potassium phosphate (pH 7.4) containing 1 mM DTT and 1 μg/ml PMSF. Protein concentrations were estimated using Bio-Rad dye reagent with gamma globulin as a standard. The His-tagged rBAL protein was further purified by Ni-NTA affinity chromatography.

rBAL assay

The BAL enzyme assay was modified from the procedure of Killenberg and Jordan (13). The BAL reaction mixture contained 20 μM CDCA, 5 mM ATP, 50 μM CoA, 5 mM $MgCl_2$, 50 mM NaF, and 2 μM [11, 12-^{3}H]CDCA (25 μCi/mmol) (Amersham) in 0.1 M Tris-HCl, pH 8.5 (total reaction volume 100 μl). The reaction mixture was incubated at 37°C for 2 min, then the rBAL enzyme was added in a 15-μl volume and the reaction mixture was incubated for 20 min. The reaction was stopped by the addition of 0.4 ml of 45% methanol-1.5% percholoric acid. In controls, the methanol/percholoric acid solution was added before the enzyme fraction. The reactions were then extracted twice with 3 ml of water-saturated diethyl ether and the amount of ^{3}H-CDCA-CoA in the aqueous phase was determined by scintillation spectrometry.

To determine the K_m of expressed rBAL for CDCA, the CDCA concentration in the assay was varied from 1 to 50 μM at constant CoA and $MgCl_2$ concentrations of 50 μM and 2.5 mM, respectively. Sf9 microsomes containing expressed rBAL were diluted with 0.1 M potassium phosphate to maintain CDC-CoA production between 5% and 10% of total CDCA in the reaction. In experiments to determine the ability of bile acids and fatty acids to inhibit rBAL activity, the nonradioactive bile acids and fatty acids were dissolved in isopropanol. The appropriate amount of bile acid or fatty acid was placed in the reaction tube and air-dried. The reaction mixture was added, thoroughly mixed, and incubated at 37°C for 2 min. The reactions were carried out and [^{3}H]CDC-CoA isolated as described previously.

Product identification

To identify the products generated by rBAL from CDCA and CoA, reactions were carried out as described previously without being stopped by the addition of the methanol/perchloric acid solution. An aliquot (30 μl) of the reaction mix was applied directly to a reverse-phase C_{18} HPLC column with a mobile-phase flow rate of 1 ml/min. The elution solvent consisted of mixtures of buffer A (5% (v/v) isopropanol/10 mM ammonium acetate, pH 7) and buffer B [40% isopropanol (v/v)/6 mM ammonium acetate, pH 7]. The gradient conditions were 0–8 min, 0–85% buffer B; 8–15 min, 85–100% buffer B; 15–30 min, 100% buffer B, and the absorbance of the eluate was monitored at 262 nm. A peak at 12.7 min appeared after injection of either chemically synthesized CDC-CoA or the reaction mixture. The peaks were collected and analyzed by electrospray ionization mass-spectrometry (ESI-MS) on a PE Sciex API III triple-quadruple mass spectrometer (Concord, Ontario, Canada). Mass spectra over the

range *m/z* 100 to 2,000 were recorded in both the positive and negative ion modes.

RESULTS

Molecular characterization of rBAL cDNA

The original partial rBAL cDNA was obtained by RT-PCR using rat liver total RNA as a template. A degenerate rBAL-selective primer was designed corresponding to part of the sequence of a peptide derived from proteolysis of pure rBAL. The degenerate rBAL primer and a dT18 oligomer were used as primers in the PCR reaction. Two major PCR products (~450 bp and 300 bp) were generated. These DNA fragments were subcloned into pCR2.1 and sequenced. One fragment, 423 bp in length, encoded the four amino acids (QKLY) immediately following the sequence of the purified peptide used to design the degenerate oligonucleotide primer. The 423 nucleotide PCR product also encoded the sequence of a separate peptide derived from purified rBAL (**Fig. 1**). The 423bp cDNA fragment was then used as a probe to screen a λZap II Sprague Dawley rat liver cDNA library to isolate the full-length rBAL cDNA.

Three separate λ cDNA clones were isolated from screening the rat liver λZAP II cDNA library using high stringency conditions. Restriction enzyme analysis and DNA sequencing showed that the clones were closely related. The nucleotide sequence and translation of the longest clone, rBAL-1, is shown in Fig. 1. Clone rBAL-2 was identical to rBAL-1 except for being 42 bp shorter at the 5′ end and truncated 9 bp prior to the polyadenylation site in rBAL-1. The third clone was identical to rBAL-1 except it lacked the initial methionine codon. The longest clone, rBAL-1, was therefore chosen for extensive sequence analysis and expression studies.

The rBAL-1 cDNA contains 51 bp of 5′-nontranslated sequence and 138 bp of 3′-nontranslated sequence with a 101 bp poly(A) tail. A putative polyadenylation signal sequence, AATAAA, is located 17 bases upstream from the beginning of the poly(A) tail. rBAL-1 encodes a protein consisting of 690 amino acids with a predicted molecular mass of 76,260 Da (Fig. 1). The translation of rBAL-1 also contains the two polypeptides derived from sequencing of peptides obtained from purified rat liver rBAL protein. The one ambiguous amino acid (aa 607) in the peptide sequences is encoded as a phenylalanine in rBAL-1. Also, the peptide generated by CNBr digestion is positioned in the translation of rBAL-1 immediately after a methionine residue.

Northern blot analysis of rBAL expression in rat tissues

Northern blot analysis of total RNA isolated from several tissues of an adult male Sprague Dawley rat was used to determine the size and tissue distribution of messages hybridizing with the rBAL cDNA. **Figure 2** shows that the rBAL cDNA detects one message, approximately 2,500 bp in length, in liver RNA. This RNA is close in size to the rBAL-1 cDNA. rBAL-1 hybridization was not detectable in RNA isolated from kidney, spleen, heart, lung, brain, or testis; however, a single slightly larger signal was observed for rat small intestine RNA.

Immunoblot analysis of rBAL

A rabbit polyclonal antibody was raised to a 463 amino acid fragment of the rBAL protein. The antibody was used in immunoblot analysis of rBAL in microsomes prepared from tissues of a young adult male Sprague Dawley rat. **Figure 3** shows that immunoreactive rBAL was readily detectable in liver microsomes but not in microsomes prepared from spleen, kidney, lung, heart, or brain. A protein migrating with a slightly lower molecular mass than liver rBAL was detected in testis microsomes although no rBAL related message was observed in Northern blot analysis of testis RNA (Fig. 2). No immunoreactive rBAL was observed in rat liver cytosol.

Expression of rBAL-1 in Sf9 insect cells

In order to characterize the enzymatic activity of rBAL, the rBAL-1 cDNA was expressed in Sf9 cells using pFastBac baculoviral vectors. rBAL-1 was expressed in both native form and with an amino terminal histidine tag. Microsomes prepared from Sf9 cells transfected with both the pFastBac1-rBAL and pFastBacHTb-rBAL vectors displayed significant amounts of rBAL activity with CDCA as a substrate (**Fig. 4**). The microsomes containing the native rBAL protein consistently displayed higher specific activities than those with the His tagged rBAL. In contrast, control Sf9 insect cell microsomes did not synthesize CDC-CoA. Analysis of the rBAL reaction by HPLC with UV detection and ESI-MS confirmed the formation of CDC-CoA. Analysis of the putative CDC-CoA product by ESI-MS showed that it had a $[M+H]^+$ molecular ion of *m/z* 1143 in the positive ion mode and a $[M\text{-}2H]^{2-}$ molecular ion of *m/z* 569 in the negative ion mode. These ions matched those observed for synthetic CDC-CoA, confirming that expressed rBAL had generated CDC-CoA.

The effect of increasing CDCA concentrations on the activity of the two expressed forms of rBAL was also determined. rBAL activity was assayed at CDCA concentrations of 1, 2, 5, 10, 20, and 50 μM. Double reciprocal plots of enzyme velocity vs. CDCA concentration, generated apparent K_m values of 32 μM for native rBAL expressed in Sf9 microsomes and 64 μM for His-tagged rBAL. Wheeler et al. (7) have reported a K_m of 18 μM for CDCA using purified rat liver BAL. These K_m values are within the normal physiological bile acid concentrations that range from 20 to 100 μM (2).

Comparison of rBAL and acyl-CoA-synthetases

Figure 5 shows the phylogenetic tree of the comparison of the translation of rBAL with the sequences of six very long-chain acyl-CoA synthetases (VLACS) and two very long-chain acyl-CoA synthetase related proteins. rBAL is 65.2–65.5% similar and 42.3–43.9% identical to VLACS proteins cloned from rat (14), mouse (15), and human (16, 17). rBAL is most homologous to a mouse VLACS-related protein (87.2% identity, 93.3% similarity) (18).

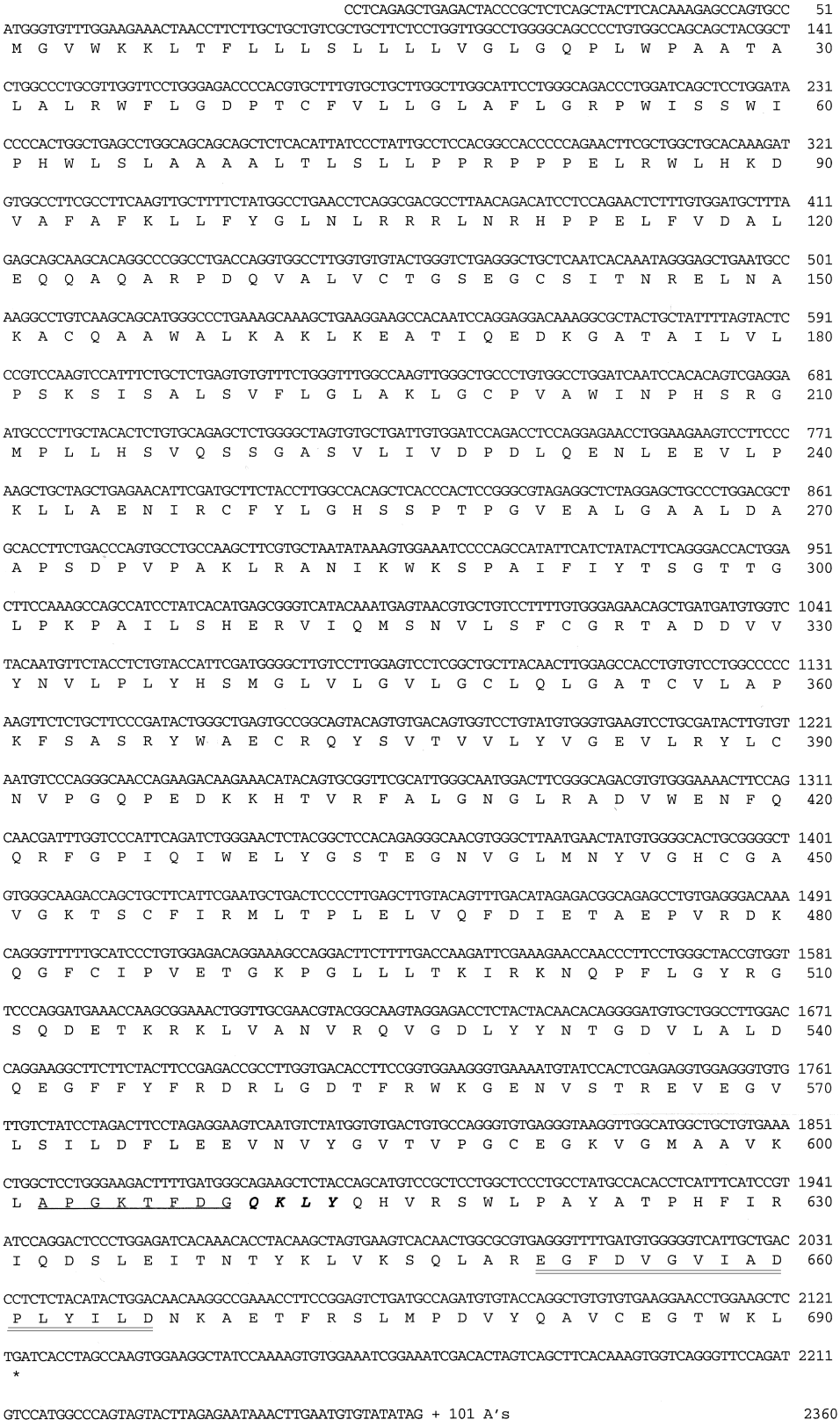

Fig. 1. Nucleotide sequence and translation of the rBAL-1 cDNA. The nucleotide sequence and the translated amino acid sequence are numbered at the right. An asterisk denotes the stop codon. The sequence of the 423 bp RT-PCR product begins at nucleotide 1856 and extends into the polyadenylation tract. The peptide sequence (aa 602–609) identified by peptide sequencing and used to construct the 5′-PCR primer is underlined. The four amino acids identified in the purified peptide and the PCR product are denoted in bold print. The sequence of a separate peptide (aa 651–666) obtained by peptide sequencing of purified rBAL is denoted by a double underlining.

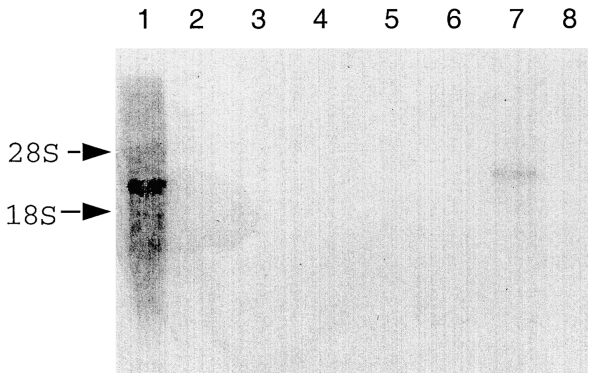

Fig. 2. Northern blot analysis of rBAL message expression in different rat tissues. Total RNA was isolated from the tissues of a male Sprague-Dawley rat using STAT-60. Five micrograms of total RNA was loaded into each lane and the RNA was resolved on a 1% agarose gel in the presence of formaldehyde and probed with ^{32}P-labeled rBAL-1 as described in Materials and Methods. RNA in lanes 1-8 were from: lane 1, liver; lane 2, kidney; lane 3, spleen; lane 4, heart; lane 5, lung; lane 6, brain; lane 7, small intestine; lane 8, testis.

High homology (71.6% identity, 85.5% similarity) is also observed with a human VLACS protein, also referred as FATP5 or hVLACS-H2 (8). Human VLACS-H2 has been reported to possess cholyl CoA synthetase activity (19) and more recently to form CoA esters of several bile acids (20). The high degree of sequence similarity between rBAL and these CoA synthetase proteins supports the inclusion of rBAL in the CoA synthetase/FATP gene family (8).

Inhibition of rBAL activity

As shown in Fig. 5, rBAL displays significant homology with the CoA synthetase or FATP gene family (8) and at least one member of this family is capable of forming cholic acid CoA esters (19). Simion et al. (21) have reported that the bile acids DCA and CA acid were substrates for rBAL activity, and Wheeler (22) has also identified LCA and THCA as substrates of rBAL purified from rat liver. Vessey et al. (6) showed activity toward CDCA, CA, DCA, and LCA with purified guinea pig BAL. Therefore, the ability of several bile acids and fatty acids to inhibit the CDC-CoA ligase activity of baculovirus expressed rBAL was investigated. **Table 1** shows that the CDC-CoA ligase activity of rBAL was strongly inhibited by LCA and moderately by THCA and DCA. CA was a relatively weak inhibitor of rBAL activity whereas norcholate did not show dose-dependent inhibition.

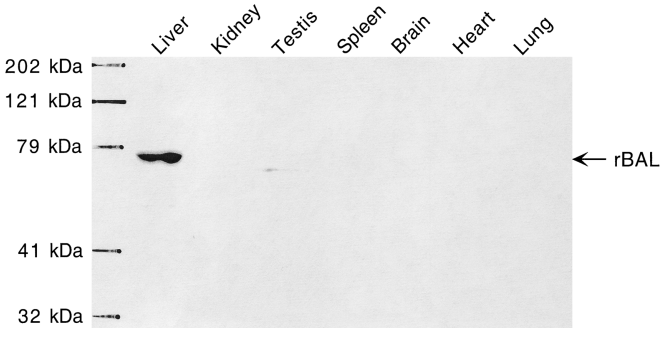

Fig. 3. Immunoblot analysis of rBAL in rat tissues. Microsomes were prepared from tissues of a young male Sprague Dawley rat and resolved by electrophoresis in 10% SDS-polyacrylamide gel. Each lane contained 80 μg of microsomal protein and the resolved proteins were electrophoretically transferred to a nitrocellulose membrane. The primary antibody was rabbit anti-rBAL antisera (1:4,000 dilution) and the secondary was goat anti-rabbit IgG horseradish peroxidase conjugate (1:60,000 dilution). Immunoconjugates were visualized using the Supersignal West Pico System (Pierce).

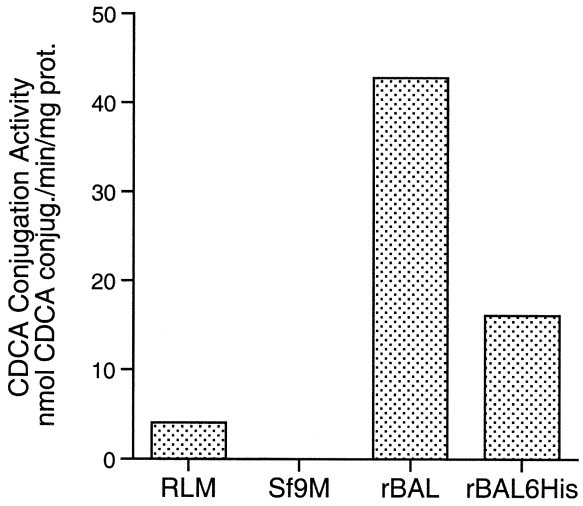

Fig. 4. Expression of rBAL activity in Sf9 insect cells. The rBAL-1 cDNA was inserted into the baculoviral vectors, pFastBac-1 and pFastBacHTb, and expressed in Sf9 insect cells. Microsomes were prepared from control cells (Sf9M) and cells infected with rBAL/pFastBac-1 (rBAL) or rBAL/pFastBacHTb (rBAL6His). Chenodeoxycholate (CDCA) conjugation activity was assayed in the microsomal samples and compared with the activity in male Sprague Dawley rat liver microsomes (RLM).

Steinberg et al. (19) have reported that human VLACS-H2, also known as FATP5, is capable of forming cholyl CoA. To determine whether fatty acids could bind and inhibit rBAL activity, several saturated and mono-unsaturated fatty acids were tested for their ability to inhibit CDC-CoA ligase activity. **Table 2** shows that the unsaturated fatty acids, myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0) did not inhibit rBAL activity. However, the mono-unsaturated fatty acids, palmitoleic acid (16:1Δ9C) and oleic acid (18:1Δ9C), displayed potent and dose-dependent inhibition of rBAL activity. In contrast, the trans-isomer of oleic acid, elaidic acid (18:1Δ9T), did not show significant inhibition, suggesting that the presence of the double bond in the 9 *cis* position is important for inhibition of rBAL activity.

Effects of detergents

In order to evaluate the importance of the lipid environment of baculovirus expressed rBAL, we investigated whether two common nonionic detergents used in the solubilization of membrane-associated proteins would affect CDC-CoA ligase activity. rBAL and increasing concentrations of detergents were pre-incubated on ice for no more

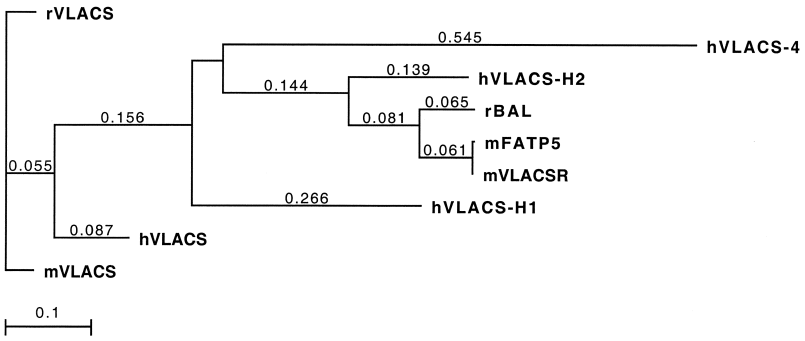

Fig. 5. Phylogenetic tree of rBAL and related proteins. The sequences of rBAL and several very long-chain acyl-CoA synthetase (VLACS) proteins were compared using the ClustalW alignment program and a phylogenetic tree generated using the McVector programs. The sequences and accession numbers of the proteins compared were rat BAL (AF242189), human VLACS-4 (AF030555), mouse VLACS-related protein (mVLACSR) (AJ223959), human VLACS-H2 (AF033031), human VLACS (D88308), human VLACS-H1 (AF064254), mouse VLACS (AJ223958), and rat VLACS (D85100).

than 10 min before the determination of enzyme activity with CDCA. A range of microsome-detergent ratios were used to include those that were utilized for solubilization of rBAL for its purification from rat liver (7). Increasing Brij-58 concentrations adversely affected rBAL CDC-CoA activity with 20% inhibition of control activity observed at 0.01 mg Brij 58/mg protein **(Fig. 6)**. In contrast, Triton X-100 had no adverse affect on rBAL activity up to 0.05 mg detergent/mg protein. However, at 0.5 mg detergent/mg microsomal protein, Triton X-100 inhibited 95% of rBAL activity as compared with 86% inhibition by Brij-58.

DISCUSSION

This report describes the cloning and expression of BAL from rat liver, the enzyme specifically responsible for the first step in the conjugation of bile acids with amino acids. The rBAL-catalyzed formation of bile acid CoA thioesters activates the bile acids. This is necessary for their subsequent conjugation with glycine or taurine. Our laboratories have previously cloned and expressed bile acid CoA:amino acid *N*-acyltransferase (BAT), the enzyme responsible for the second step in the synthesis of bile acid amino acid conjugates from both human (4) and mouse liver (10). The cloning of rBAL will allow for the further investigation of the regulation, interaction, and function of these two important enzyme systems at the molecular level.

The identity of the rBAL cDNA was confirmed by several approaches. The rBAL cDNA was isolated from a rat liver cDNA library using a cDNA probe generated by RT-PCR with rat liver total RNA as the template, according to the partial amino acid sequence of the previously purified rBAL enzyme (7). Two peptide sequences obtained from the analysis of the purified enzyme were identified in the translation of the rBAL cDNA confirming the relationship between the purified and cloned rBAL proteins. Also, high levels of CDC-CoA conjugation activity were detected in Sf9 insect cells infected with a baculoviral vector containing the rBAL cDNA, whereas no CDC-CoA conjugation activity was detected in control Sf9 cells. In addition,

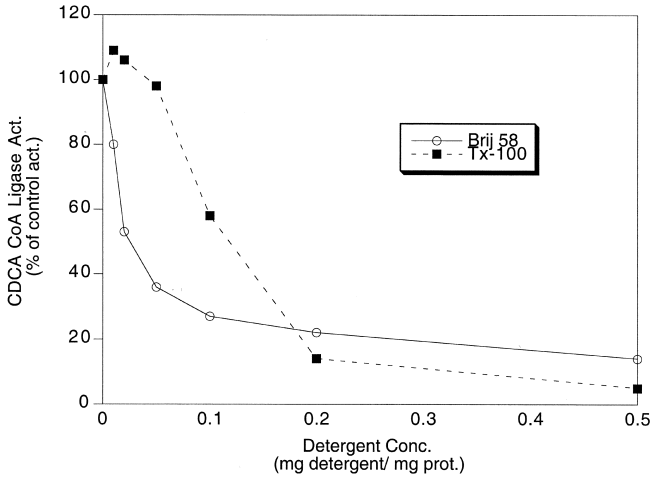

Fig. 6. Effect of detergents on rBAL activity. The effect of increasing concentrations of Brij 58 and Tx-100 on rBAL activity was measured using CDCA as a substrate. Sf9 insect microsomes containing expressed rBAL were mixed on ice with increasing ratios of detergent relative to the microsomal protein concentration. The rBAL activity in the detergent treated microsomes was then assayed with ^{3}H-CDCA as described in Materials and Methods.

TABLE 1. Inhibition of rBAL activity by bile acids

	Inhibitor Concentration		
Bile Acid	5 μM	10 μM	20 μM
Deoxycholic acid	66 ± 5	58 ± 8	40 ± 7
Lithocholic acid	13 ± 6	5 ± 5	4 ± 6
Cholic acid	83 ± 10	80 ± 11	76 ± 7
Norcholic acid	85 ± 6	80 ± 9	82 ± 7
Trihydroxycoprostanoic acid	38 ± 7	32 ± 9	26 ± 7

rBAL activity was assayed using microsomes from insect Sf9 cells containing expressed rBAL and 5 μM ^{3}H-labeled CDCA. Reactions were carried out in the presence of increasing concentrations of nonradiolabeled bile acids as described in Materials and Methods. The results are presented as percent of control reaction ± SD and represent the average of 5 or 6 reactions.

TABLE 2. Inhibition of rBAL activity by fatty acids

Fatty Acids	Inhibitor Concentration		
	0.2 μM	2.0 μM	20 μM
Myristic (14:0)	121 ± 35	127 ± 19	119 ± 39
Palmitic (16:0)	127 ± 26	132 ± 15	136 ± 18
Stearic (18:0)	129 ± 24	135 ± 27	139 ± 20
Palmitoleic (16:1Δ9C)	143 ± 42	19 ± 2	2 ± 1
Oleic (18:1Δ9C)	76 ± 7	9 ± 2	NA
Elaidic (18:1Δ9T)	109 ± 10	118 ± 16	

rBAL activity was assayed using microsomes from insect Sf9 cells containing expressed rBAL and 5 μM ^{3}H-labeled CDCA. Reactions were carried out in the presence of increasing concentrations of nonradiolabeled fatty acids as described in Materials and Methods. The results are presented as percent of control reaction ± SD and represent the average of 5 or 6 reactions. NA means that there was no detectable activity.

the amino acid sequence encoded by rBAL displays significant sequence similarity to fatty acid CoA synthetase/FATP proteins, indicating that rBAL is a member of this large gene family (23, 24).

rBAL was previously purified from rat liver microsomes and the membrane dependence of rBAL for enzymatic activity was characterized (7). Initial attempts to express active full-length rBAL in *E. coli* resulted in expression in inclusion bodies. Therefore, to increase the likelihood of detecting rBAL activity, rBAL was expressed in Sf9 insect cells using a baculoviral vector. rBAL was expressed both in its native form and with an amino terminal 6x His tag (rBAL6H). Sf9 microsomes did not form detectable levels of CDC-CoA; however, microsomes from Sf9 cells expressing either rBAL or rBAL6H displayed high levels of CDC-CoA conjugation activity. The apparent K_m of the rBAL activity for CDCA (32 μM) expressed in Sf9 cell microsomes was higher than that reported with the purified enzyme (18 μM) (7). The native enzyme purified from rat liver was solubilized with Brij-58, whereas the baculoviral expressed rBAL enzyme from Sf9 cells was assayed as a Triton-X 100 solubilized microsomal preparation. The use of different detergents could account for the discrepancy in the K_ms. Kelley and Vessey (25) have also reported that detergent mixed micelles interfere with kinetic analysis of guinea pig and porcine BAL activities. Therefore, the experiments in this report were carried out without the addition of detergents.

Amino acids 1–76 of rBAL represent a highly lipophilic region that may have a role in the association of the protein with the endoplasmic reticulum membrane. The presence of the charged His tag at the amino end of the expressed protein decreased the level of expression of rBAL in Sf9 microsomes, possibly by interfering with the membrane association of the protein. Also, the K_m of rBAL6H activity for CDCA in microsomes was approximately twice that of the native enzyme, suggesting that the 6x His tag affected the active conformation and/or membrane association of the enzyme.

Comparison of the sequence of rBAL with VLACS and related proteins from rat, mouse, and human demonstrated a high degree of similarity (Fig. 5). rBAL contains two highly conserved sequence motifs identified in the CoA synthetase gene family. The first is the AMP-binding domain (residues 294–303) that is conserved among long- and very-long chain acyl-CoA synthetases (23). The second is a domain highly conserved in the VLACS/fatty acid transport protein gene family located at amino acids 546–567 (24). These structural motifs increase the likelihood that rBAL is a member of the large fatty acid CoA synthetase/FATP gene family.

Expressed rBAL displayed high levels of bile acid conjugation activity; however, the translation of the rBAL cDNA has the most similarity to a mouse VLACS-related protein, highly expressed in liver (18). Enzymatic activity and substrates for this protein have not been reported. Another protein with a high level of similarity to rBAL is human VLACS-H2 (8). When expressed in COS-1 cells, hVLACS-H2 had low levels of activity toward several long chain fatty acids and was capable of forming cholyl CoA (19). Subsequently, hVLACS-H2 has been reported to form CoA esters of several primary and secondary bile acids as well as 3α, 7α, 12α-THCA (20). Mihalik et al. (20) have suggested that hVLACS-H2 be referred to as hBACS. These results indicate that rBAL is the rat ortholog of hVLACS.

hVLACS-H2 is selectively expressed in the microsomal fraction of human liver as would be expected of a bile acid CoA ligase (26). Polokoff et al. found fatty acid CoA ligase activity in microsomes isolated from all rat tissues; however, cholic acid CoA ligase activity was only found in liver microsomes suggesting two separate ligating enzymes (27). The possibility exists that human tissues possess multiple bile acid CoA ligases. Immunoreactive rBAL-related protein was detected in testis tissue, although the protein migrated with a different molecular mass during SDS-polyacrylamide gel electrophoresis. The identity of this protein is not known and is under investigation. Expression of VLACS-H2 in human testis tissue has not been reported.

Differences in the amino acid sequences between the VLACS and rBAL are primarily in the N terminus domain. These differences could represent targeting or signal sequences to provide localization for the protein (24). rBAL, mouse VLACS-related protein, and VLACS-H2 lack the putative carboxyl terminal peroxisomal targeting signal (-LKL) identified in the mouse and rat VLACS proteins (14, 15). Steinberg et al. (19) also reported that VLACS-H2 is expressed in the endoplasmic reticulum rather than peroxisomes. Kase and Bjorkhen (28) reported that rBAT, the second enzyme in the bile acid amidation pathway, is localized in peroxisomes and is not highly expressed in cytosol. Only one BAT gene has been identified in human (10) and mouse (4) tissues, indicating that the bile acid CoA esters may have to migrate into peroxisomes for taurine or glycine conjugation before excretion into bile, unless a bile acid CoA ligase activity is identified in peroxisomes.

The similarity between the VLACS proteins and rBAL indicates a possible overlap in function. Most of the various VLACS proteins have not been tested for their activity toward bile acids, and only VLACS-H2 has been reported

to form bile acid CoA esters as well as long chain fatty acid CoA esters (19, 20). RBAL, which is capable of conjugating several bile acids (7), was inhibited by DCA, LCA, and CA, as well as by THCA, a C_{27} bile acid precursor that is known to form a CoA ester as a step in its conversion to C_{24} bile acids (29). Norcholic acid, a C_{23} bile acid, did not inhibit rBAL, consistent with the observation that it is not metabolized to a CoA ester in vivo (30). These results indicate that rBAL is capable of synthesizing a number of different bile acid CoA esters including both C_{24} and C_{27} conjugates.

Since VLACS-H2 was reported to form both long chain fatty acid and bile acid CoA esters (19, 20), fatty acids were examined as substrates for rBAL. To investigate whether rBAL can specifically bind and/or conjugate fatty acids, several fatty acids were tested as inhibitors of rBAL catalyzed CDCA-CoA synthesis activity. rBAL was not inhibited by high concentrations of C_{14} to C_{18} saturated fatty acids (Table 2), which suggests that many saturated fatty acids are not substrates for rBAL. In contrast, rBAL was strongly inhibited by palmitoleic (C16:1Δ9C) and oleic (C18:1Δ9C) acids, each of which possess a double bond in a *cis* configuration. No inhibition was observed with elaidic acid (C18:1Δ9), which has the double bond in the trans configuration. These findings are consistent with the results reported by Vessey et al. (6), who showed that guinea pig BAL was inhibited by oleic acid. Mass spectrometry analysis and assays done with ^{3}H-labeled oleic acid revealed that oleoyl CoA and palmitoleoyl CoA were not formed and are therefore not substrates of rBAL. Even with the addition of 200 μM oleate, which is well above the K_m for other VLACS for oleoyl CoA (1.4 μM) (31, 32), no oleoyl CoA was detectable by LC-MS. Kinetic analysis showed that oleic acid inhibited rBAL activity in a mixed pattern, suggesting that palmitoleic and oleic acids may interact with the active site as well as altering the lipid environment of the protein (data not shown).

Bile acid amidation is important for increasing the fat solubilization and absorption properties of bile acids. The cloning, expression, and characterization of both enzymes involved in bile acid amidation now allows for a more complete characterization of this process. Recently, patients presenting with fat-soluble vitamin deficiencies have been diagnosed with an inability to synthesize bile acid-amino acid conjugates (33). One possibility is that the loss of bile acid amidates in these patients is related to genetic defects in the expression of BAL or BAT activity. The cloning and characterization of BAL allows for a more thorough investigation of its role in the synthesis of bile acid and fatty acid CoA esters and the interactions between these two processes.

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