Rat liver bile acid CoA:amino acid *N*-acyltransferase: expression, characterization, and peroxisomal localization

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Abstract **Bile acid CoA:amino acid *N*-acyltransferase (BAT) is responsible for the amidation of bile acids with the amino acids taurine and glycine. Rat liver BAT (rBAT) cDNA was isolated from a rat liver λZAP cDNA library and expressed in Sf9 insect cells using a baculoviral vector. rBAT displayed 65% amino acid sequence homology with human BAT (hBAT) and 85% homology with mouse BAT (mBAT). Similar to hBAT, expressed rBAT was capable of forming both taurine and glycine conjugates with cholyl-CoA. mBAT, which is highly homologous to rBAT, forms only taurine conjugated bile acids** (Falany, C. N., H. Fortinberry, E. H. Leiter, and S. Barnes. **1997. Cloning and expression of mouse liver bile acid CoA: Amino acid N-acyltransferase. *J. Lipid Res.*** 38: **86–95). Immunoblot analysis of rat tissues detected rBAT only in rat liver cytosol following homogenization and ultracentrifugation. Subcellular localization of rBAT detected activity and immunoreactive protein in both cytosol and isolated peroxisomes. Rat bile acid CoA ligase (rBAL), the enzyme responsible for the formation of bile acid CoA esters, was detected only in rat liver microsomes. Treatment of rats with clofibrate, a known peroxisomal proliferator, significantly induced rBAT activity, message, and immunoreactive protein in rat liver. Peroxisomal membrane protein-70, a marker for peroxisomes, was also induced by clofibrate, whereas rBAL activity and protein amount were not affected. In summary, rBAT is capable of forming both taurine and glycine bile acid conjugates and the enzyme is localized primarily in peroxisomes in rat liver.**—He, D., S. Barnes, and C. N. Falany. **Rat liver bile acid CoA:amino acid *N*-acyltransferase: expression, characterization, and peroxisomal localization. *J. Lipid Res.* 2003.** 44: **2242–2249.**

Supplementary key words induction • bile acid ligase • peroxisomal membrane protein-70

Amidation of bile acids in the liver with glycine or taurine prior to their excretion into bile is an important biochemical event in bile acid metabolism in humans as well as in most other mammals (1). The conjugation of bile acids with glycine or taurine significantly lowers the pKa of the unconjugated bile acids, ensuring a sustained aqueous solubility for bile acids over the wide range of pH conditions found within the enterohepatic circulation. In addition, biliary excretion of bile acids is a driving force for bile acid flow (2). Since the ability of the liver to form bile acid amidates is very high, less than 1% of the bile acids in bile under normal circumstances are unconjugated (1). Amidation of bile acids, therefore, has a fundamentally important role in biliary physiology, both in health and in disease.

Bile acids are the major metabolites of cholesterol and are synthesized in the liver. The conjugation of bile acids with glycine or 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 human BAT (hBAT) from a human liver cDNA library (4). Human BAT (hBAT) expressed in bacterial and COS cells utilizes both glycine and taurine as substrates (4). Additionally, our laboratories have cloned and expressed mouse BAT (mBAT) and determined its chromosomal location (5). Analysis of the enzymatic properties of mBAT showed that, in contrast to hBAT, mBAT is a taurine-specific conjugating form of BAT (5). There is marked species variability in the relative proportions of glycine and taurine conjugates that are present in bile due to the specific properties of BAT in each species (6) and substrate availability (7). Some species such as dogs, cats, and mice synthesize only taurine conjugates, whereas other species such as rats and humans synthesize both taurine- and glycine-conjugated bile acids (8).

Furutani et al. (9) identified a rat liver mRNA whose expression was down-regulated by systemic infection with *Escherichia coli*. Sequencing of the corresponding cDNA (termed *Kan-1*) revealed that *Kan-1* had 65% homology with hBAT. This led to the assumption that *Kan-1* was rat liver BAT (rBAT). Subsequent sequence analysis also revealed that *Kan-1* had high homology (85–86%) to mBAT (5).

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In the rat, the principal biliary bile acids are taurine conjugates with a glycine-taurine ratio approaching 1:6 (v/v) (10). This has led many investigators to assume that either taurine is the preferred amino acid substrate or that there are multiple forms of BAT with different substrate specificities. To investigate whether *Kan-1* exclusively utilizes taurine as a substrate or utilizes both taurine and glycine, the cDNA for *Kan-1* was isolated from a rat liver λZAP cDNA library and the functional enzyme expressed in Sf9 insect cells. The active enzyme was capable of forming both taurine and glycine conjugates with several bile acids. Because *Kan-1* utilizes both glycine and taurine as substrates, it is similar to hBAT and will be referred to as rBAT.

Since our laboratory has recently cloned and expressed rat liver BAL (11), we have the ability to undertake studies using rat BAL (rBAL) and rBAT to better understand the mechanism for the formation and physiological properties of bile acid conjugates and the cellular localization(s) of these enzymes. In previous studies, it has been suggested that rBAT is localized in rat liver peroxisomes (12) even though hBAT had been isolated from the cytosolic fraction of human liver (3). To confirm these previous results, rats were treated with clofibrate, a peroxisomal proliferative agent, and the effects on rBAT and rBAL expression in liver were monitored. Also, specific polyclonal antibodies and selective enzymatic assays were utilized to investigate the localization of rBAT and rBAL during the isolation of liver peroxisomes.

MATERIALS AND METHODS

Materials

Clofibrate was purchased from ICN Biomedicals Inc. (Aurora, OH). OptiPrep and tobacco etch virus (TEV) protease were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-PMP70 polyclonal antibody was obtained from Zymed Laboratories Inc. (South San Francisco, CA). [α-^{32}P]dCTP (800 μCi/mmol) and [^{3}H]glycine (10 μCi/mmol) were from Amersham Pharmacia Biotech (Piscataway, NJ). [^{3}H]taurine (29 μCi/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO). The STAT-60 RNA isolation reagent was purchased from TEL-TEST (Friendswood, TX). The expression vectors pQE-30 and pQE-31 were from Qiagen (Valencia, CA). Quickhyb was purchased from Stratagene (La Jolla, CA). The pFastBacHTb baculovirus expression system and CellFECTIN reagent were obtained from Life Technologies (Gaithersburg, MD). Cholyl CoA was synthesized from cholic acid and CoA by the method of Shah and Staple (13) with modifications as described previously (3).

Methods

Expression of rBAT in Sf9 insect cells. Due to the difficulty of expressing full-length rBAT with enzymatic activity in several prokaryotic expression vectors, the pFastBacHTb Baculovirus expression system was utilized to generate enzymatically active rBAT in Sf9 insect cells. Primers were designed according to the published rBAT sequence (9) to amplify full-length open reading frame of the rBAT from a rat liver λZap cDNA library by PCR. The forward primer (5′-TATAGAATTCAAATGGCCAAGCTGACAGCTG-3′) incorporates an *EcoR*I restriction site (underlined), and the reverse primer (5′-TATAAAGCTTCCACTCACAGCTGACTGTTG-3′) incorporates a *Hind*III restriction site (underlined) to facilitate subcloning of the rBAT sequence into the *EcoR*I-*Hind*III sites of the pFastBacHTb vector to generate an rBAT cDNA plasmid construct with a 6-His tag on the amino end that can be cleaved by TEV protease. The nucleotide sequence of rBAT is identical to the *Kan-1* sequence reported by Furutani et al. (9). DH10Bac cells were then transformed with the pFastBacHTb-rBAT plasmid for transposition of the rBAT DNA into the bacmid to form recombinant bacmids. Cells with recombinant rBAT bacmids were selected to isolate the large (>23 kb) bacmid DNA with a modified miniprep technique (Life Technologies).

To generate recombinant baculovirus particles containing rBAT, 9×10^5 Sf9 cells in mid-log phase were seeded in a 6-well plate in 2 ml/well of Sf-900 II serum-free medium (SFM) containing penicillin (50 U/ml) and streptomycin (50 mg/ml), then allowed to attach at 28°C for 1.5 h. For transfection, bacmid DNA and CellFECTIN reagent were each diluted in 100 ml Sf-900 II SFM without antibiotics, then gently mixed 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 bacmid/CellFECTIN solution diluted with 200 ml Sf-900 II SFM. The cells were incubated at 28°C for 5 h, then the diluted bacmid/CellFECTIN complexes were removed and replaced with fresh Sf-900 II SFM containing antibiotics. After incubation at 28°C for 72 h, the baculovirus remained in the supernate from each well after removal of cells by centrifugation at 500 *g* for 5 min.

To obtain cytosolic rBAT, Sf9 cells in mid-log phase in suspension culture were infected with the recombinant baculovirus and incubated in a 135 rpm orbital shaker at 28°C for 48 h. Infected cells were harvested by centrifugation at 500 *g* for 5 min. Cell pellets were resuspended in 50 mM Tris-HCl (pH 8.5) containing 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM PMSF, and 1% Nonidet P-40, and lysed by brief sonication. The cytosolic fraction was obtained by centrifugation at 100,000 *g* for 45 min. Ni-NTA affinity chromatography was performed to purify the His-tagged rBAT protein from the cytosol. The 6-His tag was subsequently cleaved by incubation with TEV protease at 4°C for 6 h. Purified rBAT was recovered from the cleavage reaction in the flow-through fraction of a second Ni-NTA affinity column. Expressed rBAT activities were measured in Sf9 cell cytosol initially to monitor expression or with purified rBAT for enzyme kinetic applications as described below.

BAT assay. rBAT activity was determined using the radioassay described by Johnson, Barnes, and Diasio (14) in which [^{3}H]amino acids are conjugated to unlabeled cholyl-CoA to form ^{3}H-labeled bile acid conjugates. The standard assay mixture contained 100 mM potassium phosphate (pH 8.4), 1.15 mM cholyl-CoA, and 0.025 μCi of the corresponding ^{3}H-labeled amino acid in a total volume of 100 μl. Reactions were initiated by the addition of cholyl-CoA, incubated at 37°C for 30 min, and terminated by addition of 1 ml 100 mM sodium phosphate (pH 2.0) containing 1% SDS. Radioactive conjugates were then extracted from unreacted labeled amino acid with water-saturated n-butanol and quantified by scintillation spectroscopy.

BAL assay. The BAL enzyme assay was modified from the procedure of Killenberg and Jordan (15). The BAL reaction mixture contained 20 μM chenodeoxycholic acid (CDCA), 5 mM ATP, 50 μM CoA, 5 mM MgCl2, 50 mM NaF, and 2 μM [11,12-^{3}H]CDCA (25 μCi/mmol) (Amersham Pharmacia Biotech) in 0.1 M Tris-HCl (pH 8.5) in a total reaction volume of 80 μl. Reactions were initiated by addition of the rBAL enzyme (microsomal fraction) in a 20 μl volume and incubated for 20 min at 37°C. Methanol-perchloric acid (45%:1.5%, v/v) was added to stop reactions. For control reactions, the methanol-perchloric acid solu-

tion was added prior to the enzyme fraction. Reactions were then extracted twice with 3 ml of water-saturated diethyl ether. The amount of [^{3}H]CDCA-CoA in the aqueous phase was determined by scintillation spectroscopy.

Kinetic experiments. Initial rates of rBAT amino acid conjugation reaction were determined at various concentrations (0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 4.0, 8.0, 10.0, and 20.0 mM) of taurine or glycine in the presence of 1.15 mM cholyl-CoA. Protein concentration and incubation time were selected so that no more than 15% of the limiting substrate was consumed in the reaction. Kinetic parameters were determined using the Enzyme Kinetics program (Trinity Software).

Preparation of rat liver subcellular fractions. Liver homogenates were prepared from young adult male Sprague Dawley rats as per the OptiPrep protocol (Invitrogen). Excised liver was transferred to ice-cold homogenization medium [0.25 M sucrose, 1 mM EDTA, 0.1% ethanol, 10 mM HEPES-NaOH (pH 7.4)], minced, and homogenized with a Potter-Elvehjem teflon-glass homogenizer. The homogenate was centrifuged at 500 *g* for 10 min. The pellet was resuspended in homogenization buffer, then rehomogenized and centrifuged. The resulting supernatant fraction was centrifuged at 4,000 *g* for 10 min to remove nuclei and heavy mitochondria. To recover the light mitochondrial fraction as well as the cellular cytosol, the supernatant fraction was centrifuged at 17,000 *g* for 15 min. This supernate was centrifuged at 100,000 *g* for 50 min to recover cytosol and the microsomal fraction. To purify peroxisomes, the light mitochondrial pellet was resuspended in homogenization solution with a Dounce homogenizer and mixed with an equal volume of OpitPrep (50% iodixanol). The suspension was centrifuged at 180,000 *g* for 3 h in a fixed-angle rotor (Beckman 50.2 Ti). Fractions were collected from a self-generated gradient by upward displacement. For analysis of subcellular rBAT and rBAL distribution, immunoblot analysis was performed with each gradient fraction as well as with the cytosolic and microsomal fractions as described below.

Immunoblot analysis of rBAT, rBAL, and PMP70. A rabbit anti-mBAT polyclonal antibody was generated using the full-length expressed mBAT protein (5). The full-length mBAT was expressed in *E. coli* M15 cells by transformation with the recombinant mBAT/pQE-30 expression vector, purified utilizing the 6-His tag, and utilized for the production of antibody. To raise the rabbit anti-rBAL polyclonal antibody, a partial-length 464 amino acid fragment of rBAL was utilized (aa 227–690) (11). This rBAL fragment was generated with an amino-terminal 6-His tag in the pQE-31 expression vector, and purified for the subsequent production of antibody. To identify the peroxisomal-containing fractions, a polyclonal rabbit antibody to peroxisomal marker protein (PMP70) was utilized (Zymed).

For immunoblot analysis, 120 μg rat liver cytosolic protein, 100 μg microsomal protein, or 80 μg rat liver subcellular fraction protein was resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk followed by incubation with a 1:1,000 dilution of rabbit anti-mBAT antibody, a 1:2,000 dilution of rabbit anti-rBAL antibody, or a 1:2,000 dilution of rabbit anti-rat PMP70 antibody. Membranes were then incubated with a 1:60,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase. Immunoconjugates were visualized using the Supersignal West Pico System (Pierce).

Northern blot analysis of rBAT mRNA expression. RNA STAT-60 was used to isolate total RNA from several different tissues (heart, liver, spleen, lung, kidney, brain, and testis) of an adult male Sprague Dawley rat. RNA (20 μg) was resolved by electrophoresis in a 1% agarose-formaldehyde gel and transferred to a nylon membrane.

To prepare probe, rBAT cDNA was amplified by PCR and purified from an agarose gel (Qiaquick Gel Extraction kit). The rBAT cDNA was labeled with [^{32}P]dCTP with the Prime It Oligo-labeling Kit (Stratagene) to a specific activity of $\sim 2.5 \times 10^6$ cpm/ng. Hybridization was carried out for 1.5 h at 68°C in Quickhyb (Stratagene). The membrane was washed twice in 2× SSC/0.1% SDS for 15 min at RT and twice in 0.1× SSC/0.1% SDS for 15 min at 60°C. Autoradiography was performed at −70°C with an intensifying screen.

RT-PCR analysis of BAT expression. The expression of BAT in rat intestinal tissue was analyzed by RT-PCR amplification of intestinal RNA. RNA was isolated from adult Sprague Dawley rats using STAT60. The RNA was converted to cDNA using a Super-Script II RT kit (Invitrogen). Amplification of BAT mRNA was carried out using Red-Taq (Sigma) at a range of temperatures in a Tgradient PCR machine (Biometra). The forward primer was 5′-ATGGCCAAGCTGACAGCTG and the reverse primer was 5′-CCACTCACAGCTGACTGTTG. Amplification of PCR products was analyzed by agarose gel electrophoresis and ethidium bromide staining.

Clofibrate induction. Young adult female Sprague-Dawley rats (178 g to 192 g) were purchased from Charles River (Wilmington, MA). Animals were allowed to acclimate in the animal care facility for 1 week and were maintained on a 12 h light/dark cycle at a temperature of 18°C to 22°C. Rats were allowed free access to food (Purina Certified Rodent Diet) and water for the duration of the study. After acclimatization, animals were randomized into experimental groups of three rats per group. For peroxisomal induction, clofibrate in corn oil was administered daily by gavage at a dose of 250 mg/kg for 7 days. Control animals received an equivalent volume of corn oil.

Preparation of rat liver cytosol and microsomes. For the preparation of cytosolic and microsomal fractions of liver, rats were weighed and then anesthetized with ether prior to surgical removal of livers, which were blotted and weighed. A portion of each liver was homogenized with a motor-driven Teflon pestle-glass homogenizer in 10 v (w/v) of ice-cold 0.07 M Tris-HCl (pH 7.4) containing 0.17 M KCl and 2 mM EDTA. The homogenate was centrifuged at 10,000 *g* for 20 min at 4°C, then the resulting supernate was centrifuged at 100,000 *g* for 50 min at 4°C to generate cytosolic and microsomal fractions. The microsomal pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 1 mM PMSF. The protein concentration of each microsomal and cytosolic fraction was determined with the Bio-Rad protein assay using a γ globulin standard then samples were aliquoted and frozen at −70°C until use.

Statistical analysis

BAL and BAT expression, immunoreactivity, and enzyme activities were expressed as means ± SD. The effect of clofibrate on these parameters was carried out using Student's *t*-test.

RESULTS

Expression of rBAT in Sf9 cells

Initial attempts to express rBAT in *E. coli* with vectors such as pMAL-p2× and pKK233-2 were unsuccessful due to proteolytic degradation of the expressed protein. In contrast, the baculovirus expression system with Sf9 insect cells generated high levels of stable rBAT expression. To evaluate rBAT expression, 200 μg of Sf9 cytosolic protein was assayed for rBAT activity under standard conditions, utilizing either 0.25 mM taurine or 0.25 mM glycine as substrate (4, 5). There was no detectable BAT activity with cholyl-CoA and either taurine or glycine in control Sf9 cy-

tosol. However, BAT activity was present with both amino acid substrates in cytosol from Sf9 cells infected with rBAT baculovirus.

To confirm that BAT activity was due to successful expression of rBAT in Sf9 cells, immunoblot analysis with the rabbit anti-mBAT antibody was carried out (**Fig. 1**). Figure 1A shows a Coomassie blue-stained gel containing an aliquot of purified native rBAT as well as rat liver cytosol, control Sf9 cytosol, and cytosol from Sf9 expressing rBAT. Immunoblot analysis of Sf9 cytosol detects rBAT protein in Sf9 cells transfected with rBAT-baculovirus but not in control Sf9 cells (Fig. 1B). Two bands in transfected Sf9 cytosol are detected that correspond to rBAT with the 6-His tag (upper band) and without the 6-His tag (lower band) due to proteolytic cleavage. The molecular mass of the lower band corresponds to that of purified rBAT (lane 4, Fig. 1B) from which the 6-His tag has been proteolytically removed. Time-dependent TEV protease digestion of Ni-NTA-purified 6-His-BAT protein resulted in generation of a protein with the same molecular mass as that observed with native rBAT (data not shown).

rBAT kinetics

Native rBAT expressed in Sf9 cells was capable of forming both taurine and glycine conjugates. Therefore, the rates of bile acid conjugation with both taurine and glycine were determined using purified expressed rBAT (**Fig. 2**). The K_m values for glycine (4.4 ± 0.1 mM) and taurine (2.0 ± 0.1 mM) cholyl CoA conjugation with expressed purified rBAT were similar to the values obtained with expressed hBAT (5.6 mM and 1.8 mM, respectively) (4). The V_{max}/K_m value for taurine is 3.3-fold greater than that of glycine, suggesting that the expressed enzyme is more efficient at taurine conjugation of cholyl CoA than glycine conjugation.

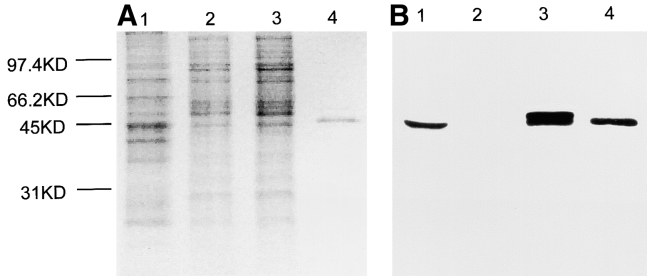

Fig. 1. Immunoblot analysis of rat liver bile acid CoA:amino acid *N*-acyltransferase (rBAT) protein expression in Sf9 insect cells. Proteins were resolved by SDS-PAGE in 12% gel. Half of the gel was stained with Coomassie blue, and the other half was electrophoretically transferred to nitrocellulose membrane and probed with a 1:1,000 dilution of rabbit anti-mouse BAT (mBAT) antibody. A: Gel stained with Coomassie blue. Lane 1, rat liver cytosol (120 μg); lane 2, normal Sf9 cell cytosol (120 μg); lane 3, cytosolic protein (120 μg) from Sf9 cells transfected with FastBac-HTb rBAT; lane 4, purified rBAT (3 μg). B: Immunoblot analysis with the anti-mBAT antibody. Lanes 1–3, identical to A; lane 4, purified rBAT (0.7 μg). Two bands are present in lane 3, which contains the expressed rBAT protein. The upper band represents 6-His-rBAT whereas the lower band represents rBAT that has lost the 6-His tag due to proteolytic cleavage.

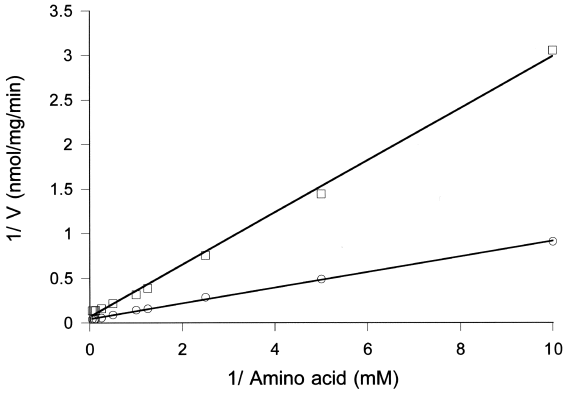

Fig. 2. Kinetic analysis of the initial reaction rate of expressed rBAT as a function of the concentration of glycine and taurine. The rates of bile acid conjugation were determined using purified rBAT (0.1 μg) in the presence of 1.15 mM cholyl-CoA with the following protein concentrations (0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 4.0, 8.0, 10.0, and 20.0 mM) of glycine (squares) and taurine (circles).

Tissue-specific expression of rBAT

To evaluate the tissue-specific expression of rBAT, cytosolic fractions of heart, liver, spleen, lung, kidney, brain, and testis tissue from a young adult male Sprague-Dawley rat were analyzed to evaluate expression of rBAT protein and message. **Figure 3A** shows an immunoblot analysis of cytosol prepared from these tissues with the specific rabbit anti-mBAT antibody. Of the tissues tested, only the liver had detectable levels of immunoreactive rBAT protein. A corresponding Northern blot was carried out with the rBAT cDNA as a probe using total RNA prepared from these same tissues (Fig. 3B). Of the tissues examined, only the liver had detectable rBAT mRNA. In subsequent experiments, the expression of BAT could not be detected in intestinal tissue by either immunoblot analysis or by RT-PCR amplification of BAT message (data not shown). Thus, results from both immunoblot and Northern blot analysis indicate that rBAT expression is specific to the liver and is not readily detectable in other tissues.

Subcellular distribution of rBAT and rBAL

To evaluate the subcellular distribution of rBAT and rBAL, immunoblot analysis of the fractions from the self-generated OptiPrep gradient designed to purify peroxisomes was performed with mBAT and rBAL antibodies. To identify fractions enriched in peroxisomes, immunoblot analysis of PMP70 was performed using a rabbit anti-PMP70 antibody. As shown in **Fig. 4**, fractions 1–4 had high levels of both rBAT and PMP70 immunoreactivity as compared with fractions 5–8. Immunoblot analysis of an aliquot of the cytosolic fraction of liver also detected both rBAT and PMP70, indicating that these proteins have a similar distribution pattern. rBAL immunoreactive protein was localized to the microsomal fraction with no obvious immunoreactivity in cytosol or peroxisomal fractions. Also, no rBAT or PMP70 immunoreactivities were detectable in liver microsomes.

Subcellular fractions from the OptiPrep peroxisomal purification gradient were also assayed for rBAL and rBAT

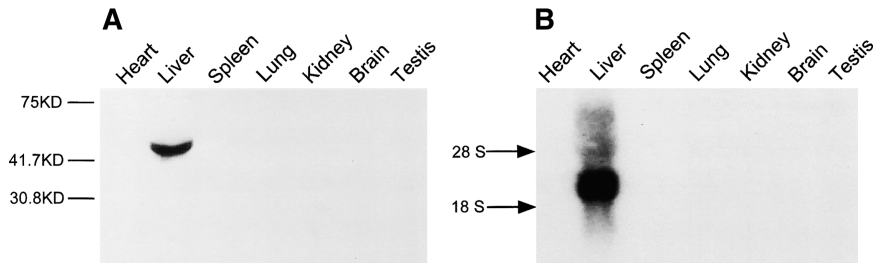

Fig. 3. Tissue-specific expression of rBAT. A: Immunoblot analysis of rBAT in different tissues. Cytosolic protein (120 μg) from several tissues (heart, liver, spleen, lung, kidney, brain, and testis) was resolved by SDS-PAGE in a 12% gel and transferred to a nitrocellulose membrane. The membrane was incubated with a 1:1,000 dilution of the rabbit anti-mBAT antibody. B: Northern blot analysis of rBAT mRNA expression in different tissues. Total RNA (20 μg) from different tissues was hybridized with a rBAT cDNA probe as described in Materials and Methods.

enzymatic activities (**Fig. 5**). rBAT activity was concentrated in fractions 1–4 with little or no activity in fractions 5–8, corresponding to the pattern of immunoreactive rBAT distribution. Consistent with the lack of rBAL immunoreactivity, rBAL activity was not found in any of the peroxisomal gradient fractions; however, rBAL activity was readily detectable in the liver microsomal fraction (data not shown).

Induction of rBAT and rBAL activities

The effect of clofibrate treatment on cytosolic rBAT and microsomal rBAL activities in rat liver was examined to determine the relationship of their expression to peroxisomal induction. For both control and clofibrate-treated rats, liver cytosolic and microsomal fractions were prepared for rBAT and rBAL activity determinations. [^{3}H]taurine and [11,12-^{3}H]CDCA-CoA were used as substrates to determine rBAT and rBAL activities, respectively. As shown in **Fig. 6**, rBAT activity was significantly increased ~2.3-fold after treatment with clofibrate ($P < 0.01$). In contrast, the activity of rBAL in microsomes was not significantly affected by clofibrate treatment. No rBAL activity was detectable in the cytosolic fraction and no rBAT activity was detected in liver microsomes following clofibrate treatment.

In addition to rBAT and rBAL activity analyses, rat liver cytosolic and microsomal fractions were evaluated for the induction of rBAT, rBAL, and PMP70 protein levels following clofibrate treatment. Immunoblot analysis was performed with the appropriate antibodies to determine the levels of the rBAT, rBAL, and PMP70 immunoreactive proteins in control and clofibrate-treated rats. As shown in **Fig. 7**, there was a significant 1.7-fold increase ($P < 0.05$) in cytosolic rBAT immunoreactive protein as well as a significant 1.7-fold increase ($P < 0.05$) in PMP70 immunoreactive protein followed by clofibrate administration. However, there were no apparent differences in microsomal rBAL levels due to treatment with clofibrate.

To examine induction of rBAT mRNA expression by clofibrate, total RNA was isolated from livers of both control and clofibrate-treated rats. Northern blot analysis with the rBAT cDNA demonstrated that mRNA in clofibrate-treated animals was expressed at a 3.1-fold higher level compared with control rats (data not shown). Similar analysis with a probe corresponding to the translated region of rBAL cDNA indicated that there was no change in

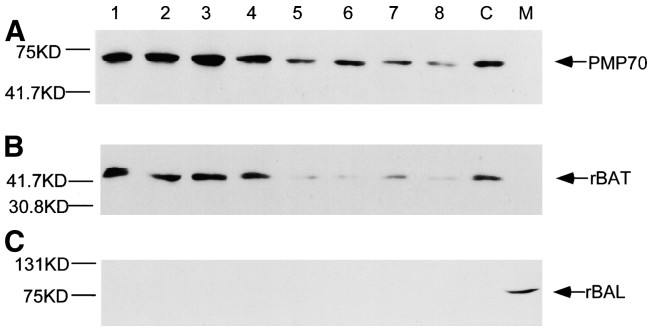

Fig. 4. Immunoblot analysis of subcellular fractions prepared from rat liver with OptiPrep. Individual lanes from left to right were loaded with 80 μg protein from fractions 1–8 from the Optiprep gradient as well as 80 μg cytosolic protein (C) and microsomal protein (M). A: Immunoreactive peroxisomal membrane protein 70 (PMP70) in liver subcellular fractions was estimated with a rabbit anti-rat PMP70 antibody. B: Immunoblot analysis of rBAT was performed with a rabbit anti-mBAT antibody. C: Immunoreactive rat bile acid CoA ligase (rBAL) was detected with a rabbit anti-rBAL antibody. Molecular mass is indicated in the left margin.

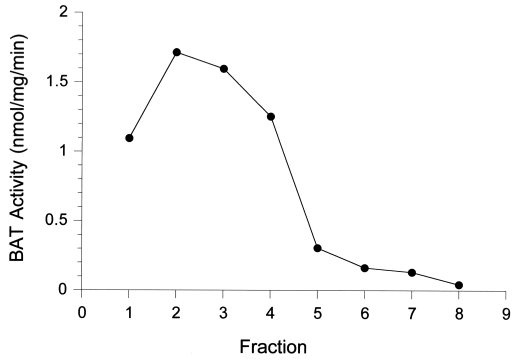

Fig. 5. BAT activity in fractions of a light mitochondrial fraction of rat liver homogenate after resolution by an OptiPrep density gradient. Fractions were collected from the gradient by upward displacement then BAT activities were assayed under standard conditions with 0.25 mM taurine as substrate. Fractions correspond to fractions in Fig. 5.

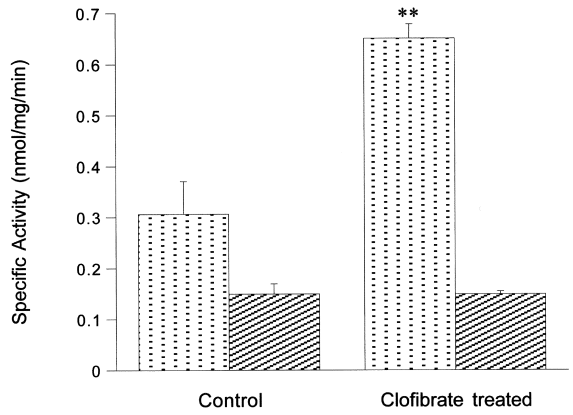

Fig. 6. Induction of liver rBAT and rBAL activities in rats treated with clofibrate. Liver cytosol and microsomes were prepared and assayed to determine rBAT activity (dotted bar) and rBAL activity (striped bar). The double asterisk indicates a significant difference in BAT activity level between clofibrate-treated and control rats ($P < 0.01$).

rBAL mRNA levels between control and clofibrate-treated animals (data not shown).

DISCUSSION

This report describes the expression, characterization, and localization of rBAT, an enzyme responsible for the amidation of bile acids in rat liver. Previously, our laboratory has cloned and expressed BAT from both human and mouse livers (4, 5). Additionally, we have cloned and expressed rBAL, the enzyme responsible for the first step in the conjugation of bile acids with amino acids, from rat liver (11). In order to further investigate the regulation, interaction, and function of the two enzymes involved in the two-step conjugation of bile acids with amino acids, rBAT has been cloned and expressed. This rat model system of rBAL and rBAT will provide the tools necessary to better investigate bile acid amidation.

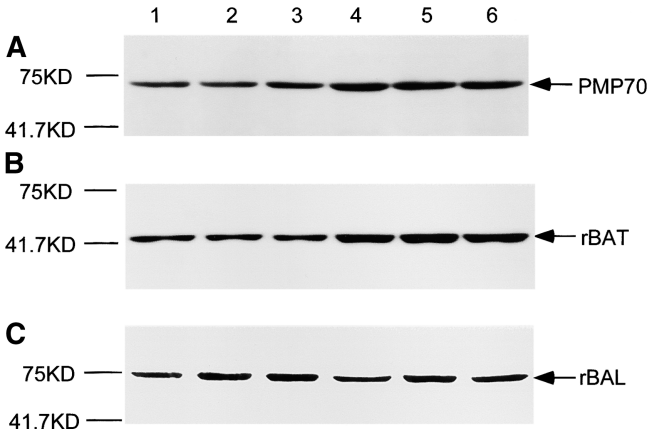

Fig. 7. Immunoblot analyses of PMP70, rBAT, and rBAL protein in clofibrate-treated rats. Immunoreactive PMP70 in liver cytosol (A), rBAT in liver cytosol (B), and rBAL in liver microsomes (C) were evaluated by immunoblot analysis of 120 μg protein using rabbit anti-rat PMP70 antibody, rabbit anti-mBAT antibody, or rabbit anti-rBAL antibody, respectively. Individual lanes 1–3 represent control rats; lanes 4–6 represent clofibrate-treated rats. Molecular mass is indicated in the left margin.

Initial attempts to express rBAT in *E. coli* using several expression vectors (pKK233-2, pMAL) were complicated by the rapid proteolytic degradation of the rBAT protein. Subsequent expression of rBAT in Sf9 cells using baculovirus resulted in high levels of intact enzyme. In addition to verification of the correct rBAT cDNA sequence, the identity of the expressed enzyme was confirmed by immunoblot analysis with an anti-mBAT antibody that cross-reacts with rBAT. Expressed rBAT demonstrated conjugation rates with both taurine and glycine amino acid substrates that were similar to those found for hBAT (4). In contrast, mBAT utilizes only taurine for bile acid amidation even though it has a high degree of homology with rBAT. These results support previous reports that the high proportion of taurine-conjugated bile acids in bile is due more to differences in the enterohepatic circulation of glycine- and taurine-conjugated bile acids than differences in substrate utilization by rBAT.

Sweeny, Barnes, and Diasio (16) showed that when collecting fresh bile, the composition of the biliary bile acid amino acid conjugates changes with time. Infusing [^{14}C]cholate into the portal vein led to the finding that the glycine-taurine ratio of the newly formed bile acid conjugates was 1:1.6 rather than the 1:6 ratio in fresh bile. Thus, availability of taurine may have an important effect on the glycine-taurine conjugate ratio (17). Gurantz and Hofmann (18) had also reported a similar shift from the formation of taurine conjugates to glycine conjugates in hamsters infused with bile acids. In addition, duodenal administration of doubly labeled [^{14}C/^{3}H]bile acids revealed that the predominance of taurine-conjugated bile acids in bile was due to a marked disparity in the stability of bile acid conjugates in the enterohepatic circulation. Taurine conjugates were absorbed and reexcreted into bile without hydrolysis, whereas 75% of the glycine conjugates were hydrolyzed in the rat intestine (18). Furthermore, the effect of dietary pectin to markedly increase the proportion of glycine-conjugated bile acids in the bile is probably due to an intestinal event altering the amount of bile acid hydrolase activity, and hence allowing an increase in recycling of intact glycine-conjugated bile acids (19).

The utilization of glycine in addition to taurine in the amidation of bile acids is a recent evolutionary event (8) and, in fact, most nonmammalian species as well as many mammals, including the cat, dog, and mouse, are unable to form glycine conjugates (8). Additionally, within the group of mammals that conjugate both taurine and glycine, the ratio of the two conjugates, as well as their respective K_ms, can vary greatly between species (6). Thus, the biochemical properties of rBAT that are similar to those of hBAT may be beneficial in applying results found with the rat bile amidation to the human situation.

Kwakye et al. (20) have previously reported low levels of BAT activity and immunoreactive protein in rat liver cytosol using a rabbit anti-hBAT antibody. The tissue-specific

expression of rBAT was explored by immunoblot analysis using the rabbit anti-mBAT antibody. Of the rat tissues investigated, only liver was positive for the presence of immunoreactive rBAT protein. Northern blot evaluation of the same tissues with a specific rBAT cDNA probe validated the immunoblot results with the detection of rBAT mRNA only in the liver. These results are consistent with the role of the liver as the primary site of bile acid amidation, although low levels of BAT expression may be present in the kidney (21). This may be due to the presence of related *N*-acyltransferases that are involved in the amino acid conjugation of xenobiotics (22, 25).

Further studies were undertaken to determine the subcellular localization of rBAT. Previous immunohistochemical studies demonstrated that BAT is present in both hepatocytes and bile ductular cells of both humans and rats (23), but the subcellular localization of this enzyme was not determined. To this end, cellular fractionation studies were undertaken with results indicating a peroxisomal localization of rBAT. Results from both enzymatic activity and immunoblot analyses were correlated to verify the occurrence of rBAT in peroxisomes. Analysis of the same fractions with an antibody to PMP70, a peroxisomal membrane protein, verified the colocalization of rBAT with PMP70. rBAT immunoreactivity was not present in the microsomal fraction of rat liver. Immunoblot analysis of these same peroxisomal and microsomal fractions with our specific rBAL antibody demonstrated the microsomal localization for rBAL (11). Very similar results describing the localization of BAT and BAL enzymatic activities in human liver subcellular fractions were reported by Solaas et al. (24). The occurrence of rBAT and PMP-70 in cytosol may be related to the release of rBAT from the peroxisomes during isolation from liver. Extensive homogenization of liver tissue involved with standard protocols for the isolation of cytosol results in the presence of most BAT protein and activity in the cytosolic fraction. Alternatively, many peroxisomal proteins are expressed in the cytosol then translocated to the peroxisomes by a distinct multistep process (25). The cytosolic localization may then be related to transport prior to peroxisomal incorporation. The conserved C-terminal amino sequence of hBAT, mBAT, and rBAT is −SQL. This is similar but not identical to the PST1 peroxisomal targeting sequence (26). Enzymes with imperfect PST1 sequences are found both in the peroxisomes and the cytosol (27).

Amidation of newly formed bile acids is proposed to occur in peroxisomes, whereas the amidation of bile acids reabsorbed in the gut occurs in the cytosol of hepatocytes (27). The presence of BAT activity in both cytosol and peroxisomes would permit conjugation of both newly formed and recycled bile acids by the same enzyme. Current evidence suggests a single BAT enzyme is present in the livers of rats, mice (5), and humans (6). Regulation of bile acid amidation would then reside with the heterogeneous family of bile acid CoA ligases that form the substrates for amino acid conjugation by BAT. This would also be consistent with the inefficient localization of BAT to the peroxisome allowing for presence of BAT in both cellular sites where bile acid conjugation occurs. Interestingly, rat BAL, which is reported to form CoA esters with C24 and C27 bile acids (11, 22), is detected only in microsomes and its expression is not affected by clofibrate treatment. This suggests that rBAL is involved in the amidation of bile acids absorbed from the portal blood supply. Mihalik et al. (28) have reported that the human homolog of rBAL (hBACS) (11) is localized in microsomes, whereas a separate very-long chain CoA synthetase (VCLS) capable of conjugating 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) but not C24 bile acids is expressed in both microsomes and peroxisomes. These authors propose that hBACS is primarily expressed in periportal hepatocytes where it functions in the reconjugation of bile acids delivered in portal blood. In humans, VCLS is then involved in the conjugation of THCA and the synthesis of C24 bile acids.

To support the peroxisomal location of rBAT, rats were treated with the peroxisome-proliferating agent clofibrate, and BAT and BAL activity and expression analyzed. As anticipated, hepatic rBAT activity was elevated in the cytosol of rats treated with clofibrate, whereas rBAL activity was unaffected. Immunoblot analysis with rBAL and rBAT antibodies confirmed these results. The effectiveness of the clofibrate treatment was confirmed by analysis of these same liver cytosols with the anti-PMP70 antibody, indicating that this peroxisomal marker protein was elevated in the clofibrate-treated rats. This result also suggests that the two enzymes in bile acid amidation are regulated independently.

The results from this study indicate that expressed rBAT is effective in the amidation of both taurine and glycine. In contrast, mBAT is capable of forming only taurine conjugates (5). The molecular or physiological rationale for this selectivity in amidate formation is not known. It has been reported that rBAT expression is regulated in pathological conditions such as sepsis and cholestasis (9), although the consequences of changes in bile acid amidation are not known. Expression and characterization of the two enzymes in the bile acid amidation pathway will permit a more detailed analysis of the function and changes in bile acid metabolism in normal and disease states.

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