The human bile acid-CoA:amino acid N-acyltransferase functions in conjugation of fatty acids to glycine

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Running title: characterization of human bile acid-CoA:amino acid N-acyltransferase

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Abbreviations: BA, bile acid; BACAT, bile acid-CoA:amino acid N-acyltransferase; BACS, bile acid-CoA synthetase; CoASH, coenzyme A; CTE-I, cytosolic acyl-CoA thioesterase I; EST, expressed sequence tag; PCR, polymerase chain reaction; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); CA, cholic acid; CA-CoA, choloxy-CoA; CDCA-CoA, chenodeoxycholoyl-CoA.
ABSTRACT

Bile acid-CoA:amino acid N-acyltransferase (BACAT) catalyzes the conjugation of bile acids to glycine and taurine for excretion into bile. By use of site-directed mutagenesis and sequence comparisons, we have identified Cys235, Asp328 and His362 to constitute a catalytic triad in human BACAT, and to identify BACAT as a member of the type-I acyl-CoA thioesterase gene family. We therefore hypothesized that hBACAT may also hydrolyze fatty acyl-CoAs and/or conjugate fatty acids to glycine. We here show that recombinant hBACAT can hydrolyze also long- and very long-chain saturated acyl-CoAs (mainly C16:0 to C26:0), and by mass spectrometry it was verified that hBACAT also conjugates fatty acids to glycine. Tissue expression studies showed strong expression of BACAT in liver, gallbladder and proximal and distal intestine, but BACAT is also expressed in a variety of tissues unrelated to bile acid formation and transport, suggesting important functions also in regulation of intracellular levels of very long-chain fatty acids. Green fluorescent protein localization experiments in human skin fibroblasts showed that the hBACAT enzyme is mainly cytosolic. Therefore, the cytosolic BACAT enzyme may play important functions in protection against toxicity by accumulation of unconjugated bile acids and non-esterified very long-chain fatty acids.

KEYWORDS

Bile acid-CoA:amino acid N-acyltransferase; bile acid; acyl-CoA thioesterase; N-acylglycines; enterohepatic circulation.
INTRODUCTION

Bile acid (BA) formation is the major pathway in mammals for excretion of cholesterol (for review, see (1)). BAs are synthesised from cholesterol in the liver and are conjugated to either glycine or taurine before secretion into the bile. This conjugation (or amidation) plays several important biological roles in that it promotes secretion of BAs and cholesterol into bile, and increases the detergent properties of BAs in the intestine, which facilitates lipid and vitamin absorption. BAs are deconjugated by bacteria in the intestine and recycled back to the liver for reconjugation. The initial steps in the biosynthesis of BAs involve oxidative modifications of the cholesterol backbone and side-chain to form dihydroxycholestanolic acid (DHCA) and trihydroxycholestanolic acid (THCA). DHCA and THCA are activated to their corresponding CoA-esters, followed by \(b\)-oxidative cleavage in peroxisomes to form chenodeoxycholoyl-CoA (CDCA-CoA) and choloyl-CoA (CA-CoA) respectively, which are substrates for conjugation to glycine or taurine (2-5). This conjugation is catalyzed by the enzyme bile acid-CoA:amino acid \(N\)-acyltransferase (BACAT), an enzyme recently implicated in inheritance of familial hypercholanemia (6). Recent data show that BACAT activity is present both in peroxisomes and in cytosol (5), suggesting the existence of two BACAT enzymes. It has been proposed that while the peroxosomal enzyme conjugates de-novo synthesized BAs, the cytosolic enzyme has a function in conjugating BAs recycled from the intestine to the liver. Further support for the existence of two pathways for conjugation of BAs comes from a recent study by Mihalik et al. They showed that human very long-chain acyl-CoA synthetase (VLCS), present in peroxisomes and the endoplasmic reticulum, primarily activates THCA, which is a precursor for de-novo synthesis of bile acids. In contrast, a homologue of this enzyme (VLCS-H2) located in the endoplasmic reticulum and referred to as bile acid-CoA synthetase (BACS), activates mainly BAs (7). It was therefore suggested that BACS activates recycled BAs for conjugation by
BACAT in the cytosol. However, to date only one BACAT enzyme has been identified and characterized. The enzyme has been purified from several species such as rat (8), bovine (9), domestic fowl (10), fish (11) and human (12), and partially purified from pig (13), canine (14), guinea pig and rabbit (15). Molecular cloning of the human BACAT showed that the enzyme can conjugate bile acids to both glycine and taurine (16).

We have recently identified and characterized a family of highly homologous acyl-CoA thioesterases, referred to as type-I acyl-CoA thioesterases, with putative localizations in peroxisomes (PTE-Ia and PTE-Ib), mitochondria (MTE-I) and cytosol (CTE-I) (17). Searches in databases and subsequent sequence alignments revealed that these acyl-CoA thioesterases show sequence homology only to BACAT from rat (18), mouse (19) and human (16), with a sequence identity of 40-45% to the type-I acyl-CoA thioesterases. Acyl-CoA thioesterases hydrolyze acyl-CoAs to non-esterified fatty acids and CoASH. By preserving a balance of acyl-CoA, free fatty acids and CoASH in the cell, acyl-CoA thioesterases directly, and indirectly via gene regulation, influence numerous cellular processes involved in lipid metabolism, for example β-oxidation and esterification of fatty acids (for review, see (20)). All members of this acyl-CoA thioesterase family contain a conserved serine-histidine-aspartic acid catalytic triad (21). Sequence alignments showed that BACAT also contains an almost identical catalytic triad, with one notable difference, the presence of a cysteine in place of the acyl-CoA thioesterase nucleophilic serine. Therefore it is likely that the cysteine is the nucleophilic residue in the BACAT enzyme and, possibly, that BACAT also amidates fatty acids.

In this study we characterize the human BACAT enzyme and show that in addition to catalyzing the conjugation of BAs, BACAT also conjugates fatty acids to glycine in vitro and that it can also act as a very long-chain acyl-CoA thioesterase. From transfection experiments
of BACAT as a fusion protein with green fluorescent protein (GFP) we also show that BACAT is mainly cytosolic. As BACAT also shows a wider tissue distribution than earlier anticipated, we propose that BACAT may also have other functions than the conjugation of bile acids to glycine.

**Experimental Procedures**

*Cloning and expression of human BACAT cDNA* The hBACAT cDNA encoding the entire open reading frame was amplified using the following primers: 5'-CATATGATCCAGTTGACAGCT-3' and 5'-CATATGGAGACATTCCGCCATG-3' with the addition of *Nde*I sites, indicated in bold. The full-length cDNA was amplified with One Step RNA PCR kit (AMV) (Takara Biomedicals) using a template of human liver total RNA. RT-PCR was performed at 58°C for 30 minutes followed by 35 cycles of 94°C for 1 min, 52°C for 30 sec and 72°C for 4 minutes. The resultant PCR product was cloned into the pET16b vector (Novegen Inc.) and expressed as outlined in (21), except that the bacteria were induced with IPTG at 30°C for 5 hours.

*Purification of wild-type and mutant hBACAT protein* For initial studies on hBACAT, the bacterial pellets were thawed in phosphate buffer (20 mM potassium phosphate, 0.5 M NaCl, pH 7.4) containing 50 mM imidazole and solubilized by sonication with 10 x 5 sec pulses at 5 sec intervals (XL2020 from Heat Systems). The bacterial suspension was centrifuged for 60 min at 35 000 x g at +4°C. The supernatant was filtered through a 0.22 μm filter, and Histagged recombinant proteins were purified using HiTrap™ chelating columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The recombinant protein was eluted stepwise with increasing imidazole concentrations. For some experiments, the bacterial pellet was
resuspended in Bugbuster protein extraction reagent (Novagen) according to the manufacturers instructions. The supernatant was filtered as described above and the protein purified using HiTrap™ chelating columns. Due to precipitation of the protein when using 0.5 M NaCl, the columns were equilibrated with phosphate buffer containing 20 mM potassium phosphate, 0.1 M NaCl and 10 mM imidazole, pH 7.4. The supernatant was mixed with the same buffer in a 1:1 ratio, loaded onto the column and the protein was eluted stepwise with increasing imidazole concentrations.

*Gel electrophoresis and protein determination* Purified recombinant proteins or bacterial protein extracts were separated by SDS/PAGE on 10% polyacrylamide gels and stained with Coomassie Brilliant Blue. Protein concentration was determined according to Bradford (22).

*Determination of hBACAT activity* Bile acid-CoA thioesterase and conjugation activity of hBACAT was measured spectrophotometrically at 412 nm using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The medium contained 200 mM potassium chloride, 10 mM HEPES and 0.05 mM DTNB (pH 7.4). For conjugation measurements, 50 mM glycine or taurine was added to the cuvette. Medium and substrate were premixed and the reaction was started with the addition of protein which allowed for the testing of non-enzymatic hydrolysis of the CoA esters. An $E_{412nm} = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$ was used to calculate the activity. Kinetic analysis was carried out using the Sigma Plot Enzyme Kinetics program. Activity measurements on CA-CoA and N-acylglycines were carried out in duplicate on three different hBACAT protein preparations, while measurements for CDCA-CoA and THCA-CoA were carried out in duplicate on two hBACAT protein preparations. Data are shown as mean ± S.E.M. or range (n=2-3).
Incubation mixtures were set up containing chenodeoxycholoyl-CoA (50 µM) or arachidoyl-CoA (40 µM), hBACAT enzyme (2-5 µg) and glycine (50 mM) in 50 mM potassium phosphate buffer, pH 8. The mixtures were incubated for 48 hours at room temperature after which they were purified using Sepac C18 columns. The Sepac C18 columns were equilibrated with 5 ml volumes of chloroform:methanol (2:1), followed by 95% methanol in water and finally water. The incubation mixtures were then loaded onto the column followed by a further washing step with 5 ml water. The column was then eluted using 5 ml methanol and the eluate was dried under nitrogen. The samples containing products from reactions with chenodeoxycholoyl-CoA and arachidoyl-CoA were reconstituted in ~50 µL methanol or tetrahydrofurane-water (1:1 v/v) respectively, of which 4-5 µL was loaded into gold coated glass capillaries. Mass spectrometry was performed on a Quattro Micro triple quadrupole mass spectrometer (Micromass, Wythenshawe, Manchester, UK) equipped with nano-electrospray ion source. Mass spectra were acquired in the negative ion mode over a mass scan range of m/z 50-1200 for 2 minutes at a scan rate of 4 s per scan.

Fasta3 (23) (European Bioinformatics Institute server; http://www.ebi.ac.uk/fasta33) was used to generate a multiple sequence alignment between hBACAT and its homologous sequences in the GenBank. Examination of the sequence alignment identified a possible cysteine-aspartic acid-histidine catalytic triad.

Point mutations were introduced by polymerase chain reaction (PCR), using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA.). The mutagenic sense oligonucleotides used are shown in Table I.
PCR reactions for single amino acid mutations were run for 16 cycles of 30 seconds at 95°C and 1 minute at 55°C, followed by 15 minutes at 68°C. The mutant plasmids were sequenced using the ABI Prism Dye Terminator Ready-Reaction Kit (Perkin-Elmer) at Cybergene AB (Novum, Sweden). The mutants were expressed, purified and activity was determined on bile acid-CoA substrates as described above for the wild type hBACAT.

Tissue expression of BACAT and BACS in human and mouse Total RNA was isolated from various mouse and human tissues using Ultraspec™ and stored at −70°C until further use. RT-PCR was carried out on 1 µg total RNA using One Step RNA PCR kit (AMV) (Takara Biomedicals), using the following oligonucleotide primers: human BACAT 5’-ACCATGATCCAGTTGACAG-3’ and 5’-GAGATTTGCTTCTTTATTTTC-3’; mouse BACAT 5’-ACCATGGCCAAGCTGACAG-3’ and 5’-TCCAGGAATATAATCAAGTCC-3’ (product sizes of 1295 and 1285 bp respectively): human BACS 5’-ATGGACTACGGGCTGATG-3’ and 5’-AACGTGCTGGTACAACTTCTC-3’ (24); mouse BACS 5’-GGATGCCCTTGCTACACTCTG-3’ and 5’-ACACAAGTACCGCAGGATTTCA-3’ (25) (product sizes of 622 and 539 bp respectively). The PCR products were analyzed by agarose gel electrophoresis. Permissions are held from local ethical committees for use of human and mouse samples.

Localization of human BACAT using green fluorescent fusion protein (GFP) The human BACAT open reading frame was cloned into the pcDNA3.1/NT-GFP vector (Invitrogen), in-frame with the GFP at the N-terminal end. Site-directed mutagenesis was carried out using the QuickChange™ Site Directed Mutagenesis kit, using the primer 5’-TCCAGATGTGACCAGTAGAACTCTAAGAAGACTAGATTTCC-3’ and its reverse complement primer, to mutate the C-terminal -SQL to –SKL. The mutated nucleotide (C to
A) is underlined in the primer sequence, and the nucleotide change was verified by sequencing. Human skin fibroblasts were grown as described (26). Fibroblasts were transfected with 10 µg hBACAT/NT-GFP plasmid and the hBACAT/NT-GFP -SKL mutant plasmid using Calcium Phosphate method as described, but without staining of the nucleus with Hoescht 33342 (26).

**RESULTS**

*Recombinant expression and characterization of hBACAT* For recombinant expression of hBACAT, we cloned the corresponding cDNA by PCR. Sequencing of the cDNA identified four nucleotide differences compared to the published sequence (16) of which two result in the following changes: Pro8Arg and Arg20Gln. The bile acid conjugation and thioesterase activities of the expressed hBACAT enzyme was tested using choloyl-CoA and chenodeoxycholoyl-CoA as substrates in the presence or absence of glycine and taurine. Non-enzymatic hydrolysis of the CoA esters did not occur (data not shown) while the thioesterase activity, measured in the absence of glycine, was about 164 nmol/min/mg protein with 50 µM CA-CoA and about 160 nmol/min/mg with 50 µM CDCA-CoA (Fig. 1a). Addition of glycine to the reaction increased the activity to about 762 nmol/min/mg with CA-CoA and to about 733 nmol/min/mg with CDCA-CoA. Thus, CA-CoA appears to be a slightly better substrate than CDCA-CoA for both the thioesterase and conjugation activities of hBACAT. Also, glycine is a slightly better substrate than taurine, at least when measured at saturating concentrations. In contrast, the hBACAT enzyme showed very low activity with THCA-CoA, both in the presence and absence of glycine. It is noteworthy that the thioesterase activity is approximately 20% of the conjugation activity with both CA-CoA and CDCA-CoA. Both the thioesterase and conjugation activities of hBACAT were confirmed by ESMS (Fig. 1b).
Identification of the putative catalytic triad in hBACAT: From examination of the sequence alignment of hBACAT and its homologues in the GenBank database, a putative catalytic triad consisting of residues Cys235, Asp328 and His362 was identified in this enzyme (Fig. 2). Following identification of the amino acids in the catalytic triad, the Cys235Ala, Asp328Ala and His362Glu mutants were generated in order to test the effects of these amino acid substitutions. The corresponding proteins were expressed and purified to near homogeneity as judged by Coomassie Brilliant Blue staining of SDS-PAGE (data not shown) and characterized enzymatically. In an experiment to measure the activity of the generated mutants, the choloyl-CoA conjugation activity (measured in the presence of 50 mM glycine) of the wild type hBACAT was about 1041 nmol/min/mg protein (Table II). However, the activities of the mutated proteins Cys235Ala, Asp328Ala and His362Gln were all less than 0.4% of the wild type activity, strongly suggesting that Cys235, Asp328 and His362 indeed constitute the active site amino acids of hBACAT. We also generated a Cys235Ser mutant, which converted the BACAT into an efficient bile acid-CoA thioesterase (data not shown).

hBACAT has both acyl-CoA thioesterase and glycine conjugation activities with long- and very long-chain saturated acyl-CoAs in vitro: Since the BACAT enzymes show a high degree of homology to the type-I acyl-CoA thioesterases, we hypothesized that the BACAT enzyme may also be active on acyl-CoAs. Bile acids are much more bulky substrates than straight long-chain acyl-CoAs and acyl-CoAs are therefore likely to be accommodated in the active site of the BACAT enzyme. The expressed hBACAT enzyme was therefore incubated with fatty acyl-CoAs (10 μM of each) in both the absence and presence of 50 mM glycine (Fig. 3). Indeed, the hBACAT enzyme showed thioesterase activity with saturated long-chain acyl-CoAs, with the highest activity seen with saturated C\textsubscript{16}-C\textsubscript{20} acyl-CoAs. Incubation of these acyl-CoAs with 50 mM glycine inconsistently stimulated the activity to some degree. However, a consistent and
larger glycine-dependent stimulation of hBACAT activity was observed with the very long-chain acyl-CoA substrates (C_{22.0}-C_{26.0}). Introduction of double bonds resulted in a strikingly lower activity (compare C_{18.1}-CoA, C_{18.2}-CoA and C_{20.4}-CoA and the corresponding saturated acyl-CoAs). In order to identify the reaction products in more detail, we incubated hBACAT with arachidoyl-CoA (C_{20.0}-CoA) as a model substrate, and analyzed the reaction products by ESMS. As shown in Fig. 4a, incubation of hBACAT with arachidoyl-CoA and glycine produced both arachidic acid as well as N-arachidoylglycine, the glycine-conjugate of arachidic acid. Incubation of hBACAT with arachidoyl-CoA in the absence of glycine produced only arachidic acid (Fig. 4b). We also confirmed the formation of N-stearoylglycine by BACAT using ESMS (data not shown). A more detailed kinetic analysis of hBACAT activity with arachidoyl-CoA resulted in a V_max of 223 nmol/min/mg and a K_m of 19.3 [M] when measured in the presence of glycine (Fig. 5). These results show that hBACAT, in addition to its bile-acid conjugating activity, also possesses thioesterase and glycine conjugating activities with long- and very long-chain acyl-CoAs, at least in-vitro. Although the activity is much lower with acyl-CoAs as compared to the activity with bile acids, it is possible that both the thioesterase and glycine conjugating activities could serve important functions in-vivo.

*Human and mouse BACAT and BACS show wide tissue expression* The novel activity of the hBACAT enzyme to hydrolyze and glycine-conjugate long- and very long-chain acyl-CoAs suggests a wider function for this enzyme also in fatty acid metabolism. Recently it was shown that the hBACS enzyme can activate both bile acids and very long-chain fatty acids to their corresponding CoA-esters, suggesting a functional link between hBACS and hBACAT (7). We therefore set out to re-investigate the tissue expression of both the BACS and BACAT enzymes by RT-PCR in human and mouse tissues. Due to restricted access to human tissues, expression of BACAT and BACS was investigated only in human liver, gallbladder mucosa and pancreas.
Interestingly, both BACAT and BACS were expressed in these tissues, showing a reasonably co-ordinate expression. In addition, database searches revealed a number of EST (Expressed Sequence Tag) clones encoding BACAT being expressed mainly in human liver as expected, but also in skin, heart, lung and pancreas. In mouse, BACAT was strongly expressed in liver, kidney, gallbladder, proximal intestine, distal intestine, and a band of the correct size was also weakly detected in adrenal, heart, lung, brain and muscle (Fig. 6b). A database search revealed two EST clones from adrenal gland, one of which was obtained from the Riken Consortium (Accession No. BB593308, Riken clone No. A330001A17). This clone was sequenced and verified to encode mouse BACAT (mBACAT). In addition, ESTs were also found from mouse gallbladder, cerebellum, aorta and thymus, demonstrating a wide tissue expression of mBACAT. In mouse, BACS was most strongly expressed in liver and gallbladder, but expression was also evident in kidney, proximal intestine, adrenal and weakly in heart and muscle. We also compared the expression of hBACAT in mouse and human liver, showing that BACAT is much more strongly expressed in mouse than in human (Fig. 6c).

**Human BACAT is localized in cytosol** The human and mouse BACAT enzymes contain a C-terminal variant -SQL of the well-characterized consensus peroxisomal type-1 targeting signal (PTS1) of -SKL (serine, lysine, leucine), the latter which has been shown to target proteins to peroxisomes (27). To establish whether BACAT is localized in peroxisomes, we cloned the human BACAT in-frame with GFP at the N-terminal end, which leaves the C-terminal -SQL sequence accessible. The plasmid encoding the hBACAT-GFP fusion protein was transfected into human skin fibroblasts, and by using immunofluorescence microscopy for detection of a Tritc labelled anti-GFP antibody, hBACAT showed a diffuse GFP expression, with little sign of a punctate pattern, indicative of a cytosolic localization (Fig. 7a). Similar results were obtained by transfection of the mouse BACAT-GFP fusion plasmid (data not shown). We also
mutated the C-terminal -SQL to -SKL, the consensus PTS1 targeting signal, and transfected this NT-GFP construct into human skin fibroblasts. Immunofluorescence microscopy showed that the hBACAT-SKL mutant was translocated to peroxisomes based on the punctate pattern (Fig. 7b). Similar results were also obtained for the mouse BACAT-SKL mutant (data not shown).

**DISCUSSION**

*Characterization of recombinant human BACAT* In this study we have cloned human BACAT, expressed the enzyme in bacteria, and purified the protein to near homogeneity for structural and biochemical characterization. The functionality of the expressed protein was assessed by activity measurements with CA-CoA and CDCA-CoA. Since the spectrophotometric assay measures conjugation activity indirectly (as release of CoASH), this activity was verified by ESMS. It has previously been shown that the substrate specificity of BACAT relates to the acyl chain length. While other bile acids such as lithocholoyl-CoA and deoxycholoyl-CoA were shown to be good substrates for the bovine BACAT enzyme, compounds such as acetyl-CoA, benzoyl-CoA and phenylacetyl-CoA proved not to be substrates (28). Also, shortening or extending the normal 4-substituted pentanoic acid side chain of CA-CoA by one methylene group, to produce norcholoyl-CoA and homocholoyl-CoA respectively, results in a severe decrease in activity (29). Our data consolidates this point as THCA-CoA proved to be a very poor substrate in comparison to CA-CoA and CDCA-CoA. The low activity with THCA-CoA is consistent with the observed accumulation of THCA in serum of Zellweger patients in which bile acid synthesis is severely impaired (30).
Identification of a catalytic triad in human BACAT It was previously confirmed by site directed mutagenesis experiments that the residue Cys235 is essential for BACAT activity (31). However, sequence alignments of BACAT to the type-I acyl-CoA thioesterases and its homologues in the databases led to the identification of a putative catalytic triad in BACAT consisting of residues Cys235, Asp328 and His362 (21). This catalytic triad was found to be conserved in human, mouse and rat BACAT sequences, in which the cysteine was located in a conserved SxCxG motif. Mutation of the Cys235, Asp328 and His362 residues leads to abolishment of BACAT activity which strongly implies that BACAT is an enzyme containing a Cys-His-Asp catalytic triad and confirms it as a member of the /[] hydrolase family. While this manuscript was in preparation another study was published, also identifying the Cys235, Asp328 and His362 as the catalytic triad amino acids in human BACAT (32).

Human BACAT can also conjugate long- and very-long chain fatty acids to glycine Although BACAT and the type-I acyl-CoA thioesterases are assumed to have completely different functions in the cell, the striking sequence and structural homology between these enzymes suggest that they probably derive from a common ancestor. The type-I thioesterases contain a nucleophilic serine in the active site where a cysteine is located in BACAT and we have previously reported an unsuccessful attempt to engineer mouse cytosolic acyl-CoA thioesterase I (CTE-I) into an acyltransferase by mutation of the serine to a cysteine (21). However, the Ser232Cys mutant of CTE-I was shown to become very strongly acylated when incubated with palmitoyl-CoA, demonstrating that replacement of Ser232 with a cysteine allows a stable acyl-enzyme intermediate to be formed. Therefore the lack of acyltransferase activity in the CTE-I Ser232Cys mutant is likely to be due to structural restraints that would not allow the CTE-I active site to accept bulky substrates and/or a second nucleophile. With this in mind, we investigated whether or not BACAT can also act as a thioesterase and, by having an active site proven to accept bulky substrates, whether BACAT could also accommodate the less bulky
fatty acyl-CoA substrates. Although BACAT contains a cysteine as the nucleophilic residue, the wild-type enzyme catalyzed the hydrolysis of CA-CoA and CDCA-CoA to the free bile acid and CoASH with an activity of approximately 20% of the conjugating activity. Interestingly, the BACAT enzyme also hydrolyzed saturated fatty acyl-CoAs of 16-26 carbons in chain-length. The activity with fatty acyl-CoAs was approximately 20% of that with CA-CoA. However, mono-, di- and polyunsaturated acyl-CoAs were much poorer substrates than the corresponding saturated acyl-CoA. Addition of glycine only had a marginal stimulatory effect on the activity with 14-18 carbon acyl-CoAs, while addition of glycine clearly stimulated the activity with C20:0-C26:0 acyl-CoAs, indicating that fatty acids may also be conjugated to glycine by the BACAT enzyme in-vitro. Analysis of incubation products by ESMS showed that BACAT indeed has the ability to conjugate fatty acids to glycine. This acyl chain-length specificity of BACAT suggests a possible function in conjugation of long- and very-long chain saturated fatty acids to glycine.

*The human BACAT enzyme is cytosolic* There has been some controversy during the past years concerning the subcellular localization of BACAT. Previous studies have reported BACAT activity in the lysosomal fraction of human liver (33), in the microsomal/peroxisomal fraction of rat liver homogenates (4,34) and guinea pigs (35), as well as the soluble fraction of liver homogenates from rat (8). Experiments carried out on homogenates from frozen human liver showed BACAT to be cytosolic, although the idea was not excluded that the freezing and thawing of the liver may have caused lysis of the cellular organelles leading to a redistribution of BACAT activity from the peroxisomes to the cytosol (13). More recently it has been shown that BACAT activity is present both in cytosolic and peroxisomal fractions (5). Although both the human and mouse BACAT proteins contain a variant -SQL (16) of the type I peroxisomal targeting signal (PTS1) –SKL (36), our data shows that the BACAT protein is mainly...
cytosolic. However we cannot exclude some very weak peroxisomal localization which is not visible due to the strong expression in cytosol. Mutation of the -SQL sequence to -SKL results in the translocation of the protein from the cytosol, exclusively to peroxisomes. These results are consistent with recent findings showing that the C-terminal –SQL is only able to interact strongly with the PTS1 receptor if it is preceded by a basic amino acid at position –4 (37). A study on human catalase demonstrated that the last four amino acids (-KANL) are necessary and sufficient to target this protein to peroxisomes (38). Mutation of the lysine at position –4 to a non-basic residue was shown to abolish targeting to peroxisomes. Our data which show that the human BACAT (ending -TSQL) and mouse BACAT (ending -SSQL) enzymes are cytosolic further underscores the importance of a basic residue at position –4 in at least some variants of the –SKL consensus PTS1.

Possible physiological functions for the cytosolic BACAT enzyme- There are two pathways for conjugation of bile acids to glycine or taurine in the cell. BACAT activity is present in peroxisomes for amidation of de-novo synthesized bile acids, while a cytosolic BACAT enzyme functions in amidation of recycled bile acids (5). The specific BACAT activity from human (5) and mouse liver (Solaas, K., Kase, B.F., Pham, V., Hunt, M.C., and Alexson S.E.H., unpublished results) is much higher in the peroxisomal fraction compared to the cytosolic fraction. Based on the present finding that BACAT is mainly cytosolic, it is proposed that the function of this BACAT is in the conjugation of recycled bile acids, and it is predicted that there exists another, as yet unidentified, peroxisomal BACAT enzyme that conjugates de-novo synthesized bile acids. Conjugation of bile acids is believed to occur mainly in the liver. In mouse, the cytosolic BACAT enzyme is strongly expressed in liver, gallbladder and intestine (both proximal and distal), compatible with an important function in amidation of bile acids and protection of gastro-intestinal mucosal cells from accumulation of free bile acids. Recycled
free bile acids need to be activated to the corresponding CoA-ester prior to conjugation to glycine by the BACAT enzyme. Recent studies have shown that bile acids are mainly activated by BACS, a microsomal bile acid-CoA synthetase, while THCA, a precursor of de-novo bile acid synthesis in peroxisomes, is mainly activated by very long-chain acyl-CoA synthetase (VLCS) located in the peroxisomal and endoplasmic reticulum membranes (7,39). Pircher et al have now shown that both BACAT and BACS are target genes of the farnesoid X receptor (FXR), the key nuclear receptor involved in regulation of bile acid sythnesis, consolidating their function in conjugation of bile acids (40). Previous studies have shown the expression of BACS (24), and the mouse homologue of BACS, the very long-chain acyl-CoA synthetase related protein (25), to be largely liver specific, but our data also shows expression in human gallbladder mucosa and pancreas. In mouse, BACS expression was strong in liver and gallbladder, consistent with a function in activation of bile acids. The novel activities of BACAT in hydrolysis of very long-chain acyl-CoAs and conjugation to glycine suggest that BACAT may have more general functions in fatty acid metabolism than was previously believed, and therefore would be also expressed in other tissues. Kwakye et al. showed the presence of both BACAT mRNA and immuno-reactive protein (13) along with BACS activity (41) in rat kidney, which is to date the only reported extra-hepatic expression of BACAT. We reinvestigated tissue expression of BACAT in more detail using RT-PCR and found strong expression in liver, kidney, gallbladder and proximal and distal intestine, with weak expression in adrenal, muscle, lung and brain. Computer searches in databases identified ESTs from liver, gallbladder, cerebellum and adrenal gland (in line with the data shown in Fig. 6), but also in aorta (four ESTs) and thymus (one EST). In human, expression was high in liver, as expected. We also analyzed four samples from gallstone patients, two of which showed cholesterolosis and two which did not. Expression of BACAT mRNA was at least as high in the gallbladder mucosa of two of the patients as in normal liver, although the different expression levels did
not appear to correlate to the different phenotypes. Expression of BACAT was also evident in pancreas. Searches in human databases identified ESTs mainly in liver, but also in lung, skin, heart and pancreas. Our data also show that BACS is expressed in other tissues than liver, i.e. in gallbladder mucosa and pancreas in human. Interestingly, BACS and VLCS also activate very long-chain fatty acids to their CoA-esters, suggesting that co-expression of the BACS and VLCS enzymes with the cytosolic BACAT could mediate conjugation of fatty acids. Although in-vivo conjugates of medium-chain fatty acids to glycine have been detected in blood of patients suffering from defects in fatty acid metabolism (42,43), only sparse information is available concerning in-vivo conjugation of long-chain fatty acids to glycine. Here we have shown that BACAT, an enzyme located in a wide number of tissues in the body, can catalyze the formation of N-acylglycines, which could provide a possible pathway for excretion of these fatty acids under conditions when they may otherwise become toxic.

To conclude we have identified Cys235, Asp328 and His362 in human BACAT as the catalytic triad amino acids, and deduced that BACAT is member of the \( \alpha/\beta \) hydrolase enzyme family. Furthermore, we show that BACAT is a mainly cytosolic enzyme which probably functions in conjugation of recycled bile acids. The novel thioesterase and glycine conjugating activities on long- and very long-chain fatty acyl-CoA substrates, together with a wide tissue expression suggests that BACAT may be involved in protection against accumulation of free bile acids and very long-chain fatty acids throughout the body.
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**FIGURE LEGENDS**

Fig. 1. *Conjugation of bile acids to glycine and taurine by recombinant hBACAT.* (a) Wild type hBACAT was expressed and purified as described under “Experimental Procedures”. hBACAT activity was measured in duplicate on 2-3 different protein preparations with chlooyl-CoA (CA-CoA), chenodeoxycholoyl-CoA (CDCA-CoA) and trihydroxycholestanoyl-CoA (THCA-CoA) at 50 μM in the absence or presence of 50 mM glycine (Gly) or taurine (Tau). Data shown are mean ± S.E.M. (n=3) for measurements on CA-CoA, and mean ± range (n=2) for measurements on CDCA-CoA and THCA-CoA . (b) Negative ion electrospray mass spectrometry analysis was carried out on incubations with hBACAT in the presence of 50 μM CDCA and 50 mM glycine. The peak at m/z 391 is chenodeoxycholic acid (CDCA) and the peak at m/z 448 is N-chenodeoxycholoylglycine. No peak corresponding to chenodeoxycholoyl-CoA could be detected, therefore an m/z range showing the reaction products is shown.

Fig. 2. *Alignment of the putative catalytic triad amino acids of BACAT and the type-I acyl-CoA thioesterases.* The mouse acyl-CoA thioesterases (mCTE-I; cytosolic acyl-CoA thioesterase I, mMTE-I; mitochondrial acyl-CoA thioesterase I and mPTE-Ia and Ib; peroxisomal acyl-CoA thioesterase Ia and Ib, respectively) contain a serine (S) residue in a GxSxG motif where a cysteine (C) residue is found in the BACAT enzymes from human (h), mouse (m) and rat (r) in an SxCxG motif. The aspartic acid (D) and histidine (H) residues are conserved between all enzymes. The numbering indicates the amino acid positions in the protein sequence.
Fig. 3. Acyl-CoA chain-length specificity of hBACAT thioesterase and conjugating activities. Wild-type hBACAT was expressed in E. coli and purified as described under “Experimental Procedures”. Thioesterase and glycine conjugating activity was measured at a substrate concentration of 10 μM in the absence and presence of 50 mM glycine. Filled bars, thioesterase activity (in the absence of glycine). Stippled bars, activity in the presence of glycine. Activity was not detectable with acyl-CoAs of 10 carbons or less. Data shown are mean ± S.E.M. (n=3) for measurements on acyl-CoAs except for measurements on C26-CoA for which the data are shown as means ± range (n=2).

Fig. 4. Electrospray mass spectrometry analysis of hBACAT fatty acid conjugating activity. Negative ion electrospray mass spectrometry analysis of hBACAT fatty acid conjugating activity was carried out on incubations containing (a) 2.5 μg hBACAT, 40 μM arachidoyl-CoA (C20-CoA) and 50 mM glycine, (b) 2.5 μg hBACAT and 40 μM aracidoyl-CoA. The peak at 327 represents a contaminant from the column, present also in incubations without BACAT protein.

Fig. 5. Kinetic characterization of hBACAT activity with arachidoyl-CoA. Wild-type hBACAT activity was measured at various concentrations of arachidoyl-CoA (C20:0-CoA) in the presence of glycine (50 mM). K_m and V_max values were calculated using the Sigma Plot Enzyme Kinetics program.

Fig. 6. Tissue Expression of human and mouse BACAT and BACS
(a) RT-PCR for BACAT and BACS was carried out on 1 μg total RNA isolated from various human tissues. Lanes marked with (*) are samples from patients suffering from cholesterolosis. (b) RT-PCR for BACAT and BACS was carried out on 1 μg total RNA
isolated from various mouse tissues. (c) Comparison of hBACAT expression by RT-PCR in human and mouse liver. Blank reactions did not contain any template RNA. DNA ladder sizes are indicated on the left side of the photographs by base pairs (bp).

Fig. 7. **hBACAT is mainly localized in cytosol.**

Human skin fibroblasts were transfected with a hBACAT-GFP plasmid (with the C-terminal sequence –SQL) and the same plasmid mutated in the C-terminal to –SKL. The cells were incubated with a rabbit GFP antibody and then with a CY3 conjugated affinity pure donkey anti-rabbit IgG and examined by immunofluorescence microscopy. **A;** Wt hBACAT (-SQL), **B;** hBACAT-SKL mutant.
Table I. Summary of the oligonucleotide primers used to create human BACAT mutants.

The primer position numbering is based on the human BACAT open reading frame (ORF) sequence (16).

| Amino Acid Substitution | Primer Position | Codon change | Primer sequence, 5’-3’- the mutated codon is underlined |
|-------------------------|-----------------|--------------|----------------------------------------------------------|
| Cys235Ala               | 688-722         | TGT - GCT    | GGG GTA GTC TCT GTA GCT CAA GGA GTA CAG ATT GG           |
| Asp328Ala               | 959 - 1004      | GAT - GCT    | CCT CTT CAT TGT AGG AGA AGG TGC TAA GAC TAT CAA CAG CAA AGC |
| His362Gln               | 1074 - 1101     | CAC - CAG    | GGG GCA GGC CAG CTG ATA GAA CCT CCC                      |
Table II. *Comparison of activity of recombinantly expressed hBACAT and mutant proteins.* Wild type and various mutants of hBACAT were expressed and purified as described under “Experimental Procedures”. The purified proteins were analyzed for bile acid-CoA conjugation activity using choloyl-CoA (25-50 μM) and glycine (50 mM).

| hBACAT Enzyme | Specific Activity (nmol/min/mg) | % Activity of wild type |
|---------------|-------------------------------|------------------------|
| Wild type     | 1041                          | 100                    |
| Cys 235 Ala   | 1.6                           | 0.15                   |
| Asp 326 Ala   | 0.7                           | 0.07                   |
| His 362 Gln   | 4                             | 0.38                   |
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Fig. 1b

N-chenodeoxycholoylglycine

Relative Intensity %

CDCA
391

m/z

448
|        | 235       | 328       | 362       |
|--------|------------|------------|------------|
| hBACAT | SVCQG      | GDK       | AGH        |
| mBACAT | SVCIIG     | DDK       | AGH        |
| rBACAT | SVCIIG     | DDK       | AGH        |
|        | 232       | 324       | 358       |
| mCTE-I | GISKG      | DHH       | AGH        |
| mMTE-I | GISKG      | DHH       | AGH        |
| mPTE-Ia| GISKG      | DHH       | TGH        |
| mPTE-Ib| GLSLG      | DHH       | TGH        |
Fig. 3

Specific Activity (nmol/min/mg)

Glycine
- + - + - + - + - + - + - + - + - + - +

Acyl-CoA
C12 C14 C16 C18 C20 C22 C24 C26 C18:1 C18:2 C20:4
Fig. 4

(a) Arachidic acid

(b) N-arachidonoylglycine

Relative Intensity % vs. m/z
Fig. 5

Specific Activity (nmol/min/mg) vs. [C20-CoA] (µM)
Fig. 6b
Fig. 6c

Ladder

Human Liver

Mouse Liver

1018 bp
1636 bp

BACAT
Fig. 7

A

hBACAT-SQL

B

hBACAT-SKL
The human bile acid-CoA: Amino acid N-acyltransferase functions in conjugation of fatty acids to glycine

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