Human and Rat Bile Acid–CoA:Amino Acid *N*-Acyltransferase Are Liver-Specific Peroxisomal Enzymes: Implications for Intracellular Bile Salt Transport

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Bile acid–coenzyme A:amino acid *N*-acyltransferase (BAAT) is the sole enzyme responsible for conjugation of primary and secondary bile acids to taurine and glycine. Previous studies indicate a peroxisomal location of BAAT in peroxisomes with variable amounts up to 95% detected in cytosolic fractions. The absence or presence of a cytosolic pool of BAAT has important implications for the intracellular transport of unconjugated/deconjugated bile salts. We used immunofluorescence microscopy and digitonin permeabilization assays to determine the subcellular location of endogenous BAAT in primary human and rat hepatocytes. In addition, green fluorescent protein (GFP)–tagged rat Baat (rBaat) and human BAAT (hBAAT) were transiently expressed in primary rat hepatocytes and human fibroblasts. Catalase and recombinant GFP-SKL and DsRed-SKL were used as peroxisomal markers. Endogenous hBAAT and rBaat were found to specifically localize to peroxisomes in human and rat hepatocytes, respectively. No significant cytosolic fraction was detected for either protein. GFP-tagged hBAAT and rBaat were efficiently sorted to peroxisomes of primary rat hepatocytes. Significant amounts of GFP-tagged hBAAT or rBaat were detected in the cytosol only when coexpressed with DsRed-SKL, suggesting that hBAAT/rBaat and DsRed-SKL compete for the same peroxisomal import machinery. When expressed in fibroblasts, GFP-tagged hBAAT localized to the cytosol, confirming earlier observations. *Conclusion:* hBAAT and rBaat are peroxisomal enzymes present in undetectable amounts in the cytosol. Unconjugated or deconjugated bile salts returning to the liver need to shuttle through the peroxisome before reentering the enterohepatic circulation. (HEPATOLOGY 2007;45:340-348.)

Bile salts are synthesized from cholesterol in the liver, after which they are secreted into bile. They serve as detergents to keep fat-soluble compounds in solution for excretion of toxins and waste products in the stool, but they also promote efficient absorption of vitamins in the intestine. In recent years, bile salts have also been recognized as important signaling molecules that regulate gene transcription through activation of nuclear hormone receptors[1,2] and may modulate signaling pathways involved in cell proliferation[3,4] and apoptosis.[5,6] Bile salts are maintained in an enterohepatic circulation: at the terminal ileum, bile salts are efficiently reabsorbed into the circulation and transported back to the liver, where they are absorbed from the portal blood by hepatocytes.[7,8]

The reabsorption of bile salts is very efficient. Approximately 95% of the bile salt pool in the intestine is transported back to the liver. The remaining 5% is lost via the feces and compensated via *de novo* synthesis in the liver. Hepatic bile salt synthesis involves the activity of at least 13 different enzymes located in the endoplasmic reticulum, mitochondria, cytosol, and peroxisomes[9] (Supplementary Fig. 1). For secretion into bile via the bile salt export pump, bile salts need to be conjugated. The final step in this process is the

Abbreviations: BAAT, bile acid–coenzyme A:amino acid N*-acyltransferase; hBAAT, human BAAT; GFP, green fluorescent protein; PTS, peroxisomal targeting signal; rBaat, rat BAAT.*

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conjugation of taurine or glycine to the side chain of the C_{24} bile salt. Bile acid–coenzyme A:amino acid *N*-acyltransferase (BAAT) is the one enzyme responsible for both types of conjugation,[10] exemplified by the absence of taurine-conjugated or glycine-conjugated bile salts in the serum of patients with familial hypercholanemia that carry a homozygous mutation in BAAT.[11] Human BAAT (hBAAT) and rat Baat (rBaat) have been reported to reside in peroxisomes with variable amounts present in the cytosol.[12-14] Consequently, it has been proposed that peroxisomal BAAT is required for conjugation of *de novo* synthesized bile salts, whereas the cytosolic pool of BAAT is required for reconjugation of deconjugated bile salts returning from the intestine.

In healthy humans, approximately one-third of the bile acid pool undergoes deconjugation by intestinal bacteria on a daily basis.[15] Significant amounts of these deconjugated bile salts are reabsorbed into the circulation, indicated by the presence of micromolar concentrations of unconjugated bile salts in serum.[16] The deconjugated bile salts are reconjugated in the liver and transported back to the bile. No significant amounts of unconjugated bile salts are detected in the bile, duodenum, or upper jejunum.[17] The efficiency of glycine/taurine-conjugation of bile salts is further substantiated by the fact that serum of patients treated with unconjugated ursodeoxycholate contain almost exclusively conjugated bile salts (99.8% of total).[18,19] This is also in line with the observation that more than 97% of ursodeoxycholate or chenodeoxycholate is amidated after a single pass through isolated perfused rat livers.[20,21] A cytosolic pool of BAAT is presumed to be responsible for conjugation of these therapeutic bile salts as well as the endogenous bile salts that are deconjugated in the intestine and return to the liver.

However, the presence of a cytosolic pool of native BAAT has been established only for approaches in which peroxisomes are isolated after mechanical breakage of rat liver tissue.[12-14] In addition, a predominant cytosolic location was detected for human BAAT fused to green fluorescent protein (GFP) after recombinant expression in human fibroblastic cells.[22] Both techniques may not reflect the *in vivo* situation; peroxisomes appear fragile during isolation procedures, and peroxisomal enzymes such as BAAT may leak from the organelles to the cytosolic fraction. Furthermore, bile salt conjugation typically occurs in hepatic cells rather than fibroblast cells, and the cellular machinery involved in BAAT sorting to peroxisomes may not be optimal in the latter cell type. Human, rat, and mouse BAAT contain a peroxisomal targeting signal (PTS) at the C-terminus consisting of a Ser-Gln-Leu sequence, which is a known variant of the typical Ser-Lys-Leu (PTS1).[23] The Gln-to-Lys change in PTS1 may affect the import efficiency into peroxisomes, but this has not yet been studied in hepatocytes. Because the subcellular location of BAAT has major implications for the intracellular transport of bile salts—especially deconjugated ones—we employed novel techniques to firmly establish the subcellular location of hBAAT and rBaat in hepatocytes.

Materials and Methods

Animals. Pathogen-free male Wistar rats (220–250 g) were purchased from Charles River Laboratories Inc. (Wilmington, MA). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Experiments were performed according to guidelines of the local committee for care and use of laboratory animals.

Primary Cells, Cell Lines, Bacterial Strains, and Culture Conditions. Primary rat hepatocytes were isolated and cultured as described.[6,24] Cryopreserved human hepatocytes were purchased from Tebu-bio (Heerhugowaard, The Netherlands), and viable cells were isolated using the hepatocyte isolation kit (Tebu-bio) according to the manufacturer's protocol and were either directly used for digitonin permeabilization assay or cultured on collagen-coated plates in modified chow medium (Tebu-bio) for immunofluorescence. Normal human foreskin fibroblasts VH25 (generous gift of Prof. H. H. Kampinga, Groningen, The Netherlands) were grown as monolayers under 5% carbon dioxide in a humidified 37°C incubator in Ham's F10 medium with 15% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 250 ng/ml fungizone (Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Human fibroblast cultures were passed once per week. *Escherichia coli* Top 10 (Invitrogen BV, Breda, The Netherlands) was used for propagation and amplification of plasmid DNA. Recombinant DNA procedures were performed as described.[25] Plasmid DNA was isolated using the EndoFree Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Plasmids. For recombinant expression of enhanced GFP–tagged rBaat and hBAAT, full-length rBaat and hBAAT were PCR-amplified from rat liver cDNA and the plasmid hBAAT (generous gift of Prof. S. Barnes, Birmingham, AL[10]), respectively. Details about the primers used for cloning are shown in Supplementary Table 2. PCR fragments were inserted into pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA) using *Hin*dIII and *Sal*I.

Transient Transfection. Human fibroblasts were transfected with SAINT-MIX (Synvolux Therapeutics BV, Groningen, The Netherlands) according to the manufacturer's instructions. Primary rat hepatocytes were transfected using electroporation according to Paquereau and Le Cam[26] using the ECM600 electroporation system (Biotechnologies and Experimental Research Inc., San Diego, CA). After electroporation, 1.25×10^6 cells were seeded in each well of a 6-well plate containing a coverslip.

After 48 hours, coverslips were removed and fixed for immunofluorescence and the cells remaining in the well were lysed for Western blot analysis.

Quantitative PCR. First-strand cDNAs from different human and rat tissues (MTC panels) were obtained from BD Biosciences Clontech, Palo Alto, CA. Messenger RNA levels of hBAAT/rBaat and β-actin were quantified using the ABI Prism 7700 (Applied Biosystems, Foster City, CA). Real-time conditions were as described in Ros et al.[19] Details about primers and probes used are depicted in Supplementary Table 1.

Protein Methods. Total protein extracts of primary hepatocytes were prepared according to Schoemaker et al.[27] Digitonin assays were performed essentially as described by Biardi and Krisans[28] with minor modifications. Immediately after isolation, primary hepatocytes were washed twice with ice-cold KH buffer (50 mM HEPES and 110 mM KOAc [pH 7.2]). The cells were then resuspended in KHM buffer (20 mM HEPES, 110 mM KOAc, and 2 mM MgOAc [pH 7.2]) to a concentration of 1.5×10^7 cells/ml. Aliquots of 100 μl of cell suspension were added to 900 μl KHM buffer containing increasing concentrations of digitonin. The final concentrations of digitonin were 0, 30, 150, 500, or 1,000 μg/ml. The cells were incubated for 5 minutes at 4°C with agitation. Cells were pelleted by centrifugation (1 minute at 3,300g in an Eppendorf centrifuge) and the supernatants were collected. The cell pellets were then resuspended in 1 ml KHM buffer containing 1,000 μg/ml digitonin resulting in complete cell permeabilization. After a 30-minute incubation on ice, the tubes were centrifuged at 15,700g in an Eppendorf centrifuge, and the supernatants, representing the "pellet" fraction, were collected. Equal volumes of supernatant and pellet fractions were analyzed via Western blotting.

Western Blot Analysis. Total cell lysates or supernatant and pellet fractions were separated via 10% SDS-PAGE[29,30] and analyzed via Western blotting according to established procedures.[29,31] Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as the standard. Antibodies used were rabbit anti-mBaat (generous gift of Prof. C. Falany, Birmingham, AL[12]), mouse polyclonal anti-human BAAT (Tebu Bio), rabbit polyclonal anti-human catalase (Calbiochem Novabiochem Corp., La Jolla, CA), mouse monoclonal anti-rabbit GAPDH (Calbiochem Novabiochem Corp.) and mouse monoclonal anti-GFP (Roche Diagnostic, Almere, The Netherlands). Horseradish peroxidase–conjugated swine anti-rabbit and rabbit anti-mouse (Dako A/S, Glostrup, Denmark) and the phototope-HRP Western Blot Detection System (Cell Signalling Technology Inc., Danvers, MA) were used for detection according to the manufacturers' protocols, and blots were exposed in a ChemiDoc XRS system (Bio-Rad, Hercules, CA). Protein band intensities were quantified using Quantity One software (Bio-Rad).

Microscopy. For immunofluorescence microscopy, cells were cultured on coverslips and fixed with 4% paraformaldehyde. After permeabilization using 1% Triton X-100, fixed cells were incubated with antibodies against Baat and/or catalase. Corresponding secondary antibodies were labeled with Alexa fluor 488 or Alexa fluor 647 (Alexis Biochemicals, Lausen, Switzerland). Images were captured with a TCS SP2/AOBS system (Leica, Heidelberg, Germany).

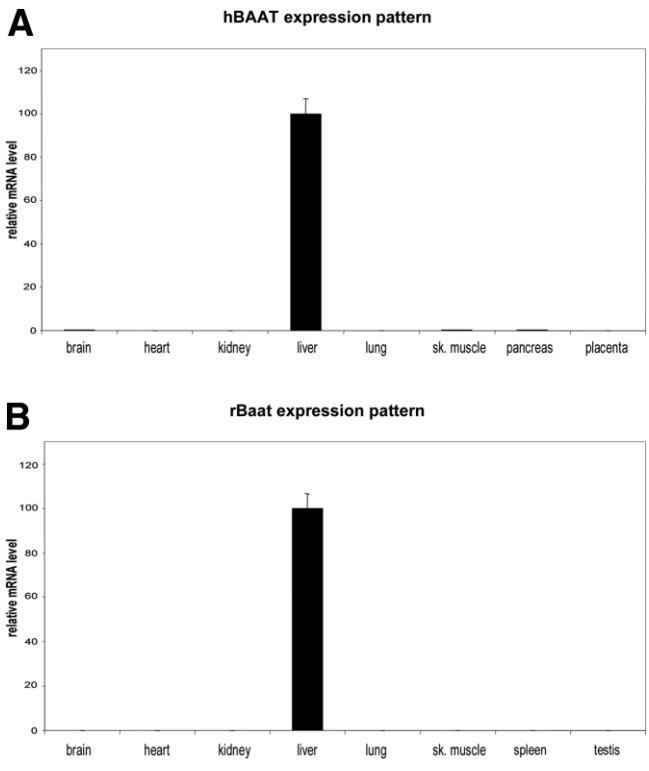

Fig. 1. BAAT is a liver-specific protein. Human (A) and rat (B) polyadenylated RNA from different tissues were subjected to quantitative real-time PCR. The relative hBAAT/rBaat messenger RNA levels were normalized to β-actin. The expression level in different tissues is expressed as a percentage of the expression in liver, but for all tissues tested this was below 1%. Bars indicate SD.

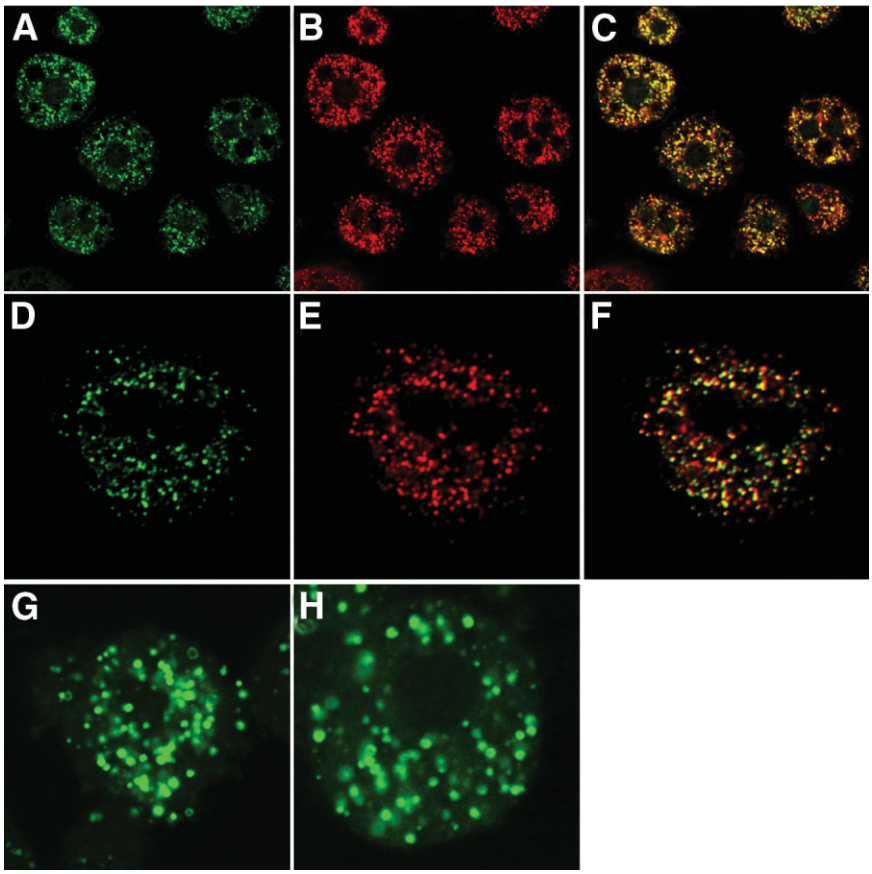

Fig. 2. Immunofluorescence microscopy for hBAAT/rBaat in human and rat hepatocytes, respectively, reveals a typical peroxisomal staining. Human hepatocytes were allowed to attach to coverslips and were immediately processed for immunofluorescence microscopy as described in Materials and Methods. hBAAT-specific antibodies revealed a typical peroxisomal staining (A,D), which was indistinguishable from the staining pattern observed when antibodies against catalase were used (B,E). The overlay images (C,F) show an almost complete colocalization of BAAT and catalase. Freshly isolated rat hepatocytes were allowed to attach to coverslips and were immediately processed for immunofluorescence microscopy as described in Material and Methods. mBAAT-specific antibodies revealed a typical peroxisomal staining (G) that was highly comparable to the staining pattern observed when antibodies against catalase were used (H). The origin of the antibodies (both raised in rabbits) did not allow us to perform colocalization studies.

Results

Liver-Specific Expression of BAAT. BAAT has been reported to be predominantly expressed in the liver.[12,32] We performed quantitative reverse-transcription PCR on a panel of different human and rat tissues to further substantiate the strict hepatic expression of hBAAT/rBaat. Both hBAAT and rBaat messenger RNA expression were only detectable in the liver (Fig. 1). Consequently, the subcellular location of the enzyme is only of physiological relevance in liver cells.

BAAT Is a Peroxisomal Protein in Hepatocytes. hBAAT and rBaat have been detected in both the peroxisome-enriched and cytosol-enriched fractions after cell fractionation procedures.[12-14] Peroxisomes are, however, known to be fragile organelles that may release some of their protein content during these procedures. Therefore, we studied the subcellular location of hBAAT/rBaat by alternative methods. First, we performed immunostaining using specific antibodies against hBAAT or rBaat on purified human and rat primary hepatocytes, respectively. For hBAAT, a dotted staining pattern was obtained with no obvious cytosolic signal (Fig. 2A,D). The dotted staining resembles the typical peroxisomal pattern observed using specific antibodies against the peroxisomal enzyme catalase (Fig. 2B,E). The overlay of both immunofluorescent staining profiles revealed that these proteins colocalize in human hepatocytes (Fig. 2C,F). The absence of a significant amount of cytosolic BAAT in human hepatocytes was particularly evident when the subcellular location of hBAAT was determined throughout the whole cell (Supplementary Fig. S2). A similar typical peroxisomal staining pattern was observed for rBaat, comparable to that of catalase (Fig. 2G,H). The nature of the antibodies (both raised in rabbit) did not allow us to perform colocalization studies for rat Baat and catalase.

To obtain quantitative data on the subcellular location of hBAAT and rBaat in human and rat hepatocytes, respectively, we exposed purified hepatocytes to increasing amounts of digitonin. Digitonin permeabilizes cellular membranes by interacting with cholesterol. Peroxisomal membranes contain low amounts of cholesterol and are therefore relatively resistant to digitonin permeabilization. Figure 3 shows that at digitonin concentrations of 30 μg/ml, the cytosolic marker protein GAPDH is released from the cells (Pellet fraction [P]) and is solely detected in the soluble fraction (S). The peroxisomal marker protein catalase remains predominantly associated with the insoluble fraction under these conditions. Raising the concentration of digitonin to 500 μg/ml results in

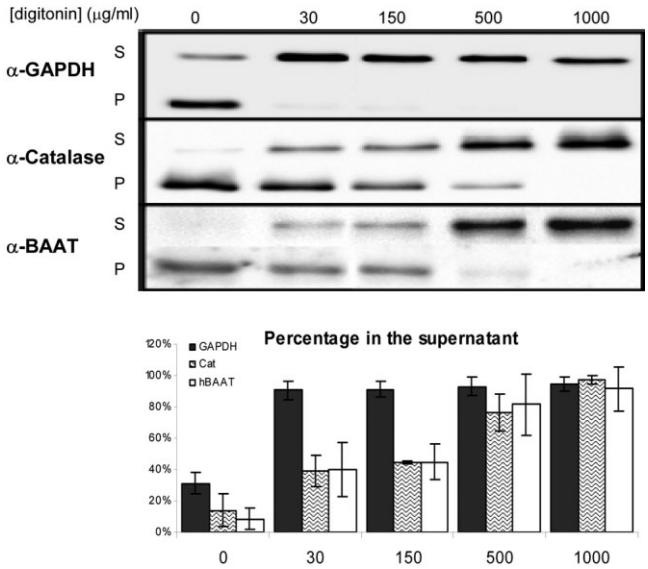

Fig. 3. Human BAAT and catalase are released from human hepatocytes at the same concentration of digitonin. Human hepatocytes were exposed to increasing concentrations of digitonin as described in Materials and Methods. Equal volumes of the samples from pellet (P) and supernatant (S) fractions were loaded, and Western blot analysis was performed with antibodies against GAPDH, catalase, and hBAAT. The upper panel shows a representative of 3 separate experiments; the lower panel shows the quantifications of the signals. Values are expressed as ratios of the total. Bars indicate SD.

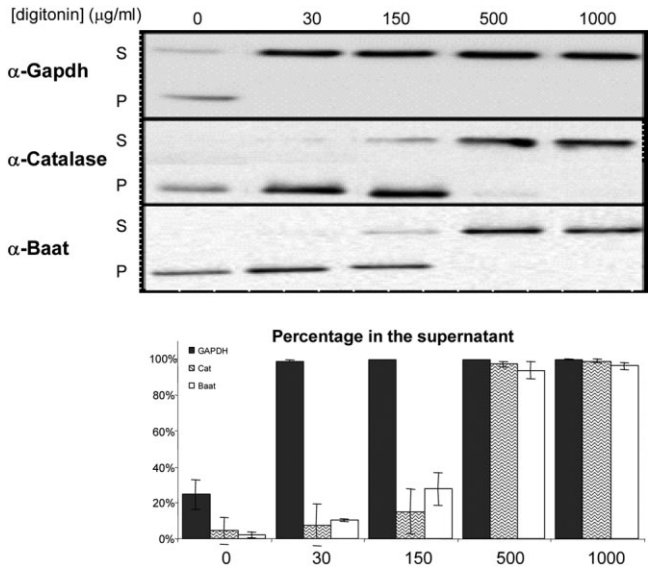

Fig. 4. Rat Baat and catalase are released from rat hepatocytes at the same concentration of digitonin. Freshly isolated rat hepatocytes were exposed to increasing concentrations of digitonin as described in Materials and Methods. Equal volumes of the samples from pellet (P) and supernatant (S) fractions were loaded, and Western blot analysis was performed with antibodies against GAPDH, catalase, and Baat. The upper panel shows a representative experiment; the lower panel shows quantifications of the signals in 3 independent experiments. Values are expressed as ratios of the total. Bars indicate SD.

an almost complete release of catalase into the soluble fraction. The release of BAAT by increasing the concentrations of digitonin mirrors the solubilization profile of catalase, suggesting that these proteins are strictly colocalizing in human hepatocytes. Essentially the same results were obtained for rBaat when purified rat hepatocytes were exposed to increasing concentrations of digitonin (Fig. 4). Again, GAPDH was released from the cellular fraction after incubation in the presence of 30 μg digitonin/ml, whereas the release profiles for catalase and rBaat were identical and only became fully solubilized at 500 μg digitonin/ml. Taken together, the microscopical and biochemical data show that both hBAAT and rBaat appear to be typical peroxisomal proteins in the liver.

Efficient Peroxisomal Accumulation of Baat-GFP Is Cell Type–Dependent. A predominant cytosolic location of human BAAT has been reported for a GFP-tagged hybrid protein expressed in human fibroblasts.[22] We confirmed this finding using fluorescence microscopy of transiently transfected human skin fibroblast with plasmids producing either GFP-hBAAT, GFP-rBaat, or GFP containing the most efficient peroxisomal targeting signal, C-terminal SKL (Fig. 5). GFP-tagged hBAAT and rBaat localized predominantly to the cytosol and no significant colocalization with catalase was detected (Fig. 5A-C) (data not shown for GFP-rBaat). In contrast, GFP-SKL was efficiently sorted to peroxisomes as demonstrated by full colocalization with catalase (Fig. 5D-F). The same GFP-hBAAT– and GFP-rBaat–expressing plasmids were used to transiently transfect freshly isolated primary rat hepatocytes. Expression of GFP-tagged hBAAT and rBaat was detected via Western blotting using antibodies against BAAT and GFP (Fig. 6A). Confocal laser scanning microscopy revealed specific and efficient accumulation of the GFP-tagged proteins in subcellular structures (Fig. 6B-D) that were positively identified as peroxisomes

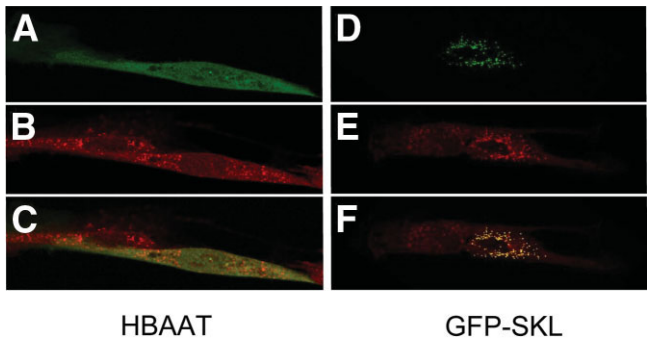

Fig. 5. GFP-tagged BAAT is predominantly cytosolic in human fibroblasts. Primary human skin fibroblasts were transfected with either GFP-hBAAT (A) or GFP-SKL (D) and stained for the peroxisomal marker catalase (B,E). GFP-hBAAT is cytosolic, and the combined image (C) does not show any colocalization. In contrast, GFP-SKL is observed in a punctuate staining only (D) that colocalizes with catalase (F).

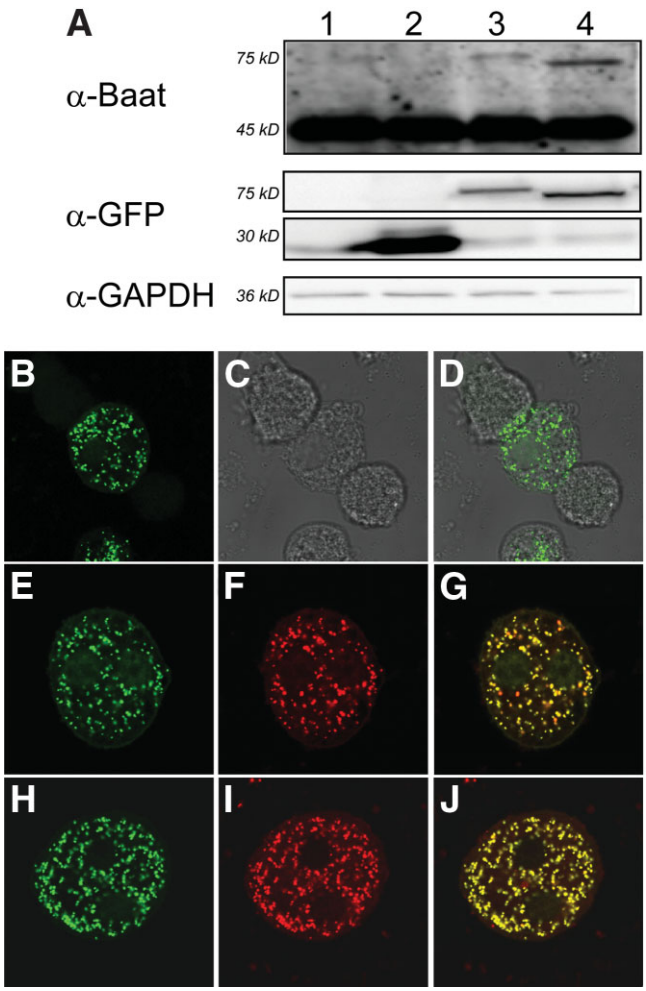

Fig. 6. Expression and subcellular location of GFP-tagged hBAAT/rBaat in rat hepatocytes. (A) Western blot analysis of rat hepatocytes transiently transfected to express DsRed-SKL (lane 1), GFP-SKL (lane 2), GFP-hBAAT (lane 3), and GFP-rBaat (lane 4). Specific antibodies raised against mBaat (top panel), GFP (middel panel), and GAPDH (loading control, lower panel) were used to detect specific protein expression. Anti-Baat detects both the endogenous rBaat as well as the heterologously expressed GFP-tagged BAAT with a molecular weight of approximately 75 kDa. Anti-GFP reveals the expression of GFP-SKL (≈30 kDa) and the GFP-BAAT/Baat proteins. (B-J) Primary rat hepatocytes transfected with either GFP-rBaat (B-D, H-J) or GFP-hBAAT (E-G) and costained for the peroxisomal marker catalase (F,I). (B,E,H) GFP-dependent fluorescent signal. (C) Bright field image of panel B. (D) Combined images of panels B and C. (F,I) Localization of catalase using anti-catalase antibodies. (G) Combined images of panels E and H. (J) Combined images of panels H and I. Both hBAAT and rBaat accumulate efficiently in peroxisomes, and the combined images (G,J) show an almost complete colocalization of BAAT and catalase.

by costaining for catalase (Fig. 6E-J). Transient transfection resulted in variable levels of expression of the introduced GFP-tagged proteins. Typical peroxisomal staining was observed for GFP-hBAAT/rBaat at all expression levels observed. Only in cells showing very high GFP-hBAAT/rBaat fluorescence was cytosolic staining also detected (data not shown). Similar results were obtained for hepatocytes expressing GFP tagged with the typical peroxisomal targeting signal SKL. Taken together, these data show that efficient targeting to peroxisomes is cell type–dependent and is most efficient in physiologically relevant cells (i.e., hepatocytes).

GFP-hBAAT with DsRed Leads to Cytosolic Baat. To compare the targeting efficiency of hBAAT with an SKL-containing protein, we cotransfected rat hepatocytes with GFP-hBAAT and DsRed-SKL. When expressed alone, GFP-BAAT is solely observed in peroxisomes (Fig. 6). However, when expressed together with DsRed-SKL, GFP-BAAT was observed predominantly in the cytosol, together with variable amounts in the nucleus (Fig. 7). At the same time, DsRed-SKL efficiently accumulated in peroxisomes. This suggests that GFP-Baat and DsRed-SKL compete for the same import machinery and that the efficiency of sorting is determined by the targeting signal.

Discussion

In this study, we show that both hBAAT and rBaat are typical peroxisomal enzymes. This subcellular location has important consequences for intracellular transport of unconjugated and deconjugated bile salts, because they have to pass through the peroxisomal compartment before secretion into bile and entry (or re-entry) into the enterohepatic circulation.

BAAT is responsible for both glyco-conjugation and tauro-conjugation of bile salts. This is a unique feature that is exemplified by the complete absence of conjugated bile salts in serum of familial hypercholanemia patients carrying a mutation in the *BAAT* gene.[11]

A peroxisomal location of rat and human BAAT has been reported previously, but always in combination with a significant cytosolic portion of this enzyme. The advantage of a dual location of BAAT in peroxisomes and the cytosol can be easily envisioned. The final steps of *de novo* bile salt synthesis (e.g., β-oxidation of the side chain)

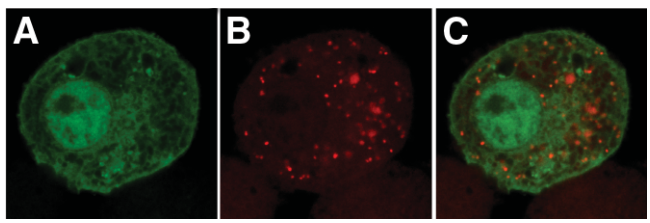

Fig. 7. Cotransfection of GFP-hBAAT and DSRed-SKL leads to cytosolic BAAT. Rat hepatocytes were transfected with both GFP-hBAAT and DsRed-SKL. When expressed together with DsRed-SKL, GFP-rBaat (A) was observed predominantly in the cytosol, together with variable amounts in the nucleus. At the same time, DsRed-SKL (B) efficiently accumulated in peroxisomes. The overlay image (C) shows little to no colocalization of the 2 recombinant proteins.

from cholesterol occur in the peroxisome, and peroxisomal BAAT is responsible for the conjugation of these newly synthesized bile salts. In the intestine, variable amounts of bile salts are deconjugated through the action of bacteria. These deconjugated bile salts recycle through the enterohepatic circulation. In the liver, they are reactivated with coenzyme A and reconjugated to taurine or glycine. A cytosolic pool of BAAT could be responsible for this reaction. However, our results refute this model and suggest a hitherto underestimated role of peroxisomes.

The existence of a significant cytosolic pool of Baat was suggested from organelle purification experiments as well as recombinant expression of GFP-tagged BAAT in fibroblasts.[13,22] Peroxisomes are fragile organelles that are known to release significant amounts of their enzymatic content during procedures based on osmotic lysis or mechanical breakage of cells.[33,34] Consequently, a certain amount of peroxisomal enzymes will "contaminate" the cytosolic fraction. Yoshihara et al.[35] have shown that the extent of leakage into the cytosol is also enzyme-dependent. In a single experiment, 75% of a true peroxisomal enzyme (e.g., ICD2) may arise in the cytosolic fraction, whereas others remain more (D-amino acid oxidase, L-hydroxyacid oxidase and, catalase) or even fully (urate oxidase) associated with the peroxisomal fraction. Consequently, it is not possible to correct for peroxisome breakage based on one selected protein. Recombinant expression of GFP-tagged BAAT also suggested a predominantly cytosolic location of BAAT.[22] We confirmed these results by expressing GFP-tagged rBaat and hBAAT in cultured skin fibroblasts. However, when the same GFP-tagged proteins were produced in primary rat hepatocytes, only a peroxisomal location was observed. Because BAAT expression was exclusively observed in the livers of humans and rats, the subcellular location of BAAT in this organ is of physiological importance. Endogenous BAAT was also found to be strictly associated with peroxisomes in hepatocytes. As an alternative to organelle purification procedures, we made use of differential permeabilization of cellular membranes by digitonin. Peroxisomal membranes contain low levels of cholesterol, and as a result resist digitonin concentrations that do permeabilize the plasma membrane. Using this assay, hBAAT/rBaat was shown to remain largely cell-associated at concentrations that fully release the cytosolic marker (GAPDH). Quantitatively, digitonin-induced release of hBAAT/rBaat from hepatocytes followed the same profile as the peroxisomal marker, catalase.

hBAAT and rBaat contain an established variant of PTS-1 (C-terminal SKL) at their C-termini, SQL. This signal is recognized by the cytosolic PTS-1 receptor Pex5p, which is responsible for sorting to the peroxisome. Degenerate forms of PTS-1 may show reduced sorting efficiency and accumulate in the cytosol. Nevertheless, it is important to note that proteins with exposed PTS-1 never lose their ability to be imported into peroxisomes, because this targeting signal is not processed during the import process, and even fully folded PTS-1–containing proteins remain importable into peroxisomes.[36,37] The efficiency of import is therefore dependent on (1) the affinity of Pex5p for the specific PTS-1 signal present in the protein,[38,39] (2) the level of Pex5p and accessory components of the import machinery, and (3) the amount of other PTS-1–containing proteins that compete for Pex5p-dependent import. Obviously, the latter 2 conditions may vary between cell types and environmental conditions. The sorting efficiency of hBAAT and rBaat indeed showed a strong dependence on cell type. Whereas GFP-tagged BAAT was fully incorporated in peroxisomes in primary rat hepatocytes, the same proteins were almost exclusively cytosolic in human skin fibroblasts. Nevertheless, GFP with the typical PTS-1 SKL at its C-terminus (GFP-SKL) was sorted efficiently to fibroblast peroxisomes. The fact that BAAT competes with other PTS-1–containing proteins for the same import route was demonstrated by coexpressing GFP-BAAT with DsRed-SKL in rat hepatocytes. When expressed alone, both proteins sort efficiently to peroxisomes, whereas in cells expressing both recombinant proteins, DsRed is peroxisomal, but clear cytosolic accumulation of GFP-BAAT is observed.

Taken together, our data show that, within the limits of detection of the methods applied in this study, hBAAT and rBaat are typical peroxisomal enzymes in the liver.

The peroxisomal location of BAAT has important implications for intrahepatocyte transport of bile salts. Bile salts entering the liver via the portal vein may have been deconjugated in the gut. These bile salts need to be reconjugated before they reenter the enterohepatic circulation. The peroxisomal location of BAAT requires import of these deconjugated bile salts into the peroxisome, thereby introducing a novel transmembrane transport process in the enterohepatic circulation of bile salts. This is a very efficient process, because unconjugated bile salts fed to or infused into rats or mice turn up as conjugated bile salts in the bile even after a single pass through the liver.[20,21] Our results suggest that unconjugated bile salts need to be taken up in peroxisomes, whereas conjugated bile salts need to be released from peroxisomes before they can be secreted into bile. The consequences of impaired transport of bile salts into and/or out of the peroxisomes

remain hypothetical. It may lead to accumulation of bile salt intermediates and therefore resemble bile salt synthesis defects. Bile salts may also accumulate to such high levels inside peroxisomes that they will damage the organelle, a phenotype that resembles peroxisome deficiency disorders such as Zellweger syndrome. Further investigation will be needed to identify and characterize these transporters and therefore enhance our understanding of the role of peroxisomes in bile salt homeostasis.

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