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New insights into bile acid amidation

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Introduction

De-novo bile acid (BA) formation is the major pathway in mammals for excretion of cholesterol (for review, see [1]). BAs are synthesized 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: 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 the intestinal flora 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 dihydroxycholestanoic acid (DHCA) and trihydroxycholestanoic acid (THCA). DHCA and THCA are activated to their corresponding CoA-esters, followed by □-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 (or amidation) 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 peroxisomal enzyme conjugates de-novo synthesized BAs, the cytosolic enzyme has a function in conjugating BAs recycled from the intestine via the enterohepatic circulation to the liver. Mihalik et al also recently suggested the existence of two pathways for conjugation of BAs. 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].

Recently it has been shown that recombinantly expressed human BACAT can also conjugate fatty acyl-CoAs to glycine [17].

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, PTE-Ib and PTE-Ic), mitochondria (MTE-I) and cytosol (CTE-I) [18, 19]. Database searches and subsequent sequence alignments revealed that these acyl-CoA thioesterases show sequence homology only to BACAT from rat [20], mouse [21] 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 □-oxidation and esterification of fatty acids (for review, see [22]).

We have now identified two new enzymes related to both BACAT and the type-I acyl-CoA thioesterases. These gene products show approximately 55% sequence identity to the mouse BACAT and approximately 95% sequence identity to eachother. Both these proteins contain a peroxisomal type-I targeting signal of -SKL, which targets the proteins to peroxisomes. We suggest that one of these proteins could be a novel peroxisomal BACAT.

Experimental procedures

Preparation and characterization of mouse liver subcellular fractions

Homogenates were prepared from SV/129 male mouse fresh liver as described [23]. Purified peroxisomes were isolated by density gradient centrifugation of light-mitochondrial fractions prepared from homogenates of pooled livers from untreated and WY-14,643 treated mice as described [24]. Bile acid-CoA:amino acid *N*-acyltransferase activity was measured as previously described (3).

Antibody production and affinity purification

A peptide with the amino acid sequence NH_2-CAAQEHSWKEIQKFLK-COOH, based on the mouse BACAT amino acid sequence [25], (with a cysteine added at the NH_2-terminal end for coupling of the peptide) was synthesized and used to

raise antisera in rabbits (Sigma Genosys). The BACAT antibody was purified from rabbit serum using a peptide affinity column prior to Western blotting.

Western blot analysis

Western blot analysis was performed as described previously [26] using the BACAT antibody and a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody. The signal was detected using enhanced chemiluminescence (ECL) (Amersham Life Science, Arlington Heights, IL) and the filters were exposed to X-ray film.

Northern blot analysis

Total RNA was prepared from mouse livers using QuickPrep^{R} total RNA extraction kit (Amersham Pharmacia Biotech, Uppsala, Sweden), and Northern blot analysis was carried out as described previously [26]. The filter was probed with a ^{32}P-labelled full-length cDNA probe for the newly identified mouse peroxisomal BACAT and the blot was exposed to X-ray film at –70°C.

Localization of mouse BACAT using green fluorescent fusion protein (GFP) -

The mouse BACAT open reading frame [25] 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 QuikChange Site Directed Mutagenesis kit (Stratagene), to mutate the C-terminal -SQL to –SKL. The nucleotide change was verified by sequencing. The open reading frame of one of the newly identified peroxisomal BACAT enzymes, ending –SKL, was cloned in-frame into the pcDNA3.1/NT-GFP vector (Invitrogen) with the GFP at the N-terminal end. Human skin fibroblasts from a healthy control and a Zellweger patient were grown as described [27]. Fibroblasts were transfected with 10 g of BACAT/NT-GFP plasmids using Calcium Phosphate method as described, but without staining of the nucleus with Hoescht 33342 [27].

Results

Mouse BACAT is regulated by treatment with WY-14,643

BACAT activity was measured in both cytosolic and peroxisomal fractions from both untreated mouse liver and those treated with the peroxisome proliferator

WY-14,643. In the cytosolic fraction, BACAT activity was approximately 20 nmol/min/mg and was unchanged by treatment with WY-14,643 (Fig. 1A). However, in peroxisomal fractions, the specific activity of BACAT was approximately 100 nmol/min/mg and was reduced to approximately 7 nmol/min/mg following treatment with WY-14,643. Western blot analysis using an antibody to the C-terminal end of the published mouse BACAT [25] showed that the BACAT protein in cytosol was also unchanged following WY-14,643 treatment, however in peroxisomes, BACAT protein was severely decreased following WY-14,643 treatment (Fig. 1B). This suggested the existence of two distinct BACAT enzymes, one in cytosol, which is not regulated by fibrate treatment, and one in peroxisomes that is highly downregulated via fibrates.

Mouse BACAT is a cytosolic enzyme

The human and mouse BACAT enzymes previously cloned [25, 28] contain a C-terminal -SQL which is a variant 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 [29]. To establish whether BACAT is localized in peroxisomes, we cloned the mouse BACAT in-frame with GFP at the N-terminal end, which leaves the C-terminal -SQL sequence accessible. The plasmid encoding the mouse BACAT-GFP fusion protein was transfected into human skin fibroblasts, and by using immunofluorescence microscopy for detection of a Tritc labelled anti-GFP antibody, mouse BACAT showed a diffuse GFP expression, with no sign of a punctate pattern, indicative of a cytosolic localization (Fig. 2A). We also mutated the C-terminal -SQL to -SKL, the consensus PTS1 targeting signal, and transfected this construct into human skin fibroblasts. Immunofluorescence microscopy showed that the mouse BACAT-SKL mutant was translocated to peroxisomes based on the punctate pattern (Fig. 2B). Similar results were obtained by transfection of the human BACAT-GFP fusion plasmid [17].

Identification of new candidate genes for peroxisomal BACAT

Database searches were carried out using the open reading frame for the mouse BACAT [25] and type-I acyl-CoA thioesterases [18] (www.ncbi.nih.gov). Two candidate genes were identified, one of which is localized on the same

chromosome as the mouse BACAT previously cloned, mouse chromosome 4. These gene products encode proteins of approximately 46 kDa, and show about 55% sequence identity to the mouse BACAT. The 2 newly identified putative BACAT enzymes show approximately 95% sequence identity to eachother.

Characterization of the new peroxisomal BACAT enzymes

The putative peroxisomal BACAT enzymes both contain an –SKL at their carboxyterminal ends, which should result in localization of these proteins in peroxisomes. To establish whether one of these BACAT proteins is localized in peroxisomes, we cloned the putative peroxisomal BACAT in-frame with GFP at the N-terminal end, which leaves the C-terminal -SKL sequence accessible. The GFP fusion protein plasmid was transfected into human skin fibroblasts from a control and a Zellweger patient, who are unable to import peroxisomal matrix proteins. Using immunofluorescence microscopy for detection of a Tritc labelled anti-GFP antibody, the peroxisomal BACAT showed a distinct punctate pattern of expression, indicative of a peroxisomal localization (Fig. 3A). In Zellweger fibroblasts, the GFP fusion protein showed a diffuse GFP expression, with little sign of a punctate pattern, indicative of a cytosolic localization (Fig. 3B)

Northern blot analysis using a full-length probe to one of the newly identified putative peroxisomal BACAT enzymes (which will recognize both peroxisomal BACAT transcripts due to the very high degree of sequence identity to eachother (95%)) showed that these enzymes are mainly expressed in liver, kidney and gallbladder, with one of these enzymes also weakly expressed in proximal and distal intestine and spleen (Fig. 4).

Discussion

There are two pathways for conjugation of bile acids to glycine or taurine in the cell. BACAT activity is present in peroxisomes for amidation (conjugation) 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 [24] is much higher in the peroxisomal fraction compared to the cytosolic fraction. Based on the findings that BACAT is mainly cytosolic in mouse

(this study) and human [17], it is proposed that the function of the previously characterized BACAT is in the conjugation of recycled bile acids, and it was predicted that there exists another 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 [17]. Recycled free bile acids need to be activated to the corresponding CoA-ester prior to conjugation to glycine or taurine 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, 30]. 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 [31].

There has been some controversy during the past years concerning the subcellular localization of BACAT. Experiments carried out on homogenates from frozen human liver showed BACAT to be cytosolic, although it was suggested that freezing and thawing of the liver may have caused lysis of cellular organelles leading to a redistribution of BACAT activity from the peroxisomes to the cytosol [13]. Immunohistochemical analysis showed that BACAT was localized in the cytoplasm of hepatocellular carcinoma cells as well as the hepatocytes and the bile duct cells in human liver [32]. 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, 25] of the type I peroxisomal targeting signal (PTS1) –SKL, our data shows that the mouse BACAT protein is mainly cytosolic. Mutation of the -SQL sequence to -SKL results in the translocation of the protein from the cytosol, exclusively to peroxisomes. The human BACAT also shows a cytosolic localization and mutation of the -SQL to –SKL results in a peroxisomal localization [17]. Therefore the previously cloned mouse [25] and human [28] BACAT enzymes are cytosolic, which suggests that there exists a further peroxisomal BACAT responsible for the conjugation of de-novo synthesized bile acids in peroxisomes.

Recent data from Inoue et al [33] further supported the hypothesis for a second peroxisomal BACAT enzyme, using the null mouse model of the hepatocyte nuclear factor 4α (HNF-4α). The HNF-4α is a nuclear receptor that has an important role in regulating expression of many liver-specific genes. In the HNF-4α-null mouse model, expression of BACAT is almost undetectable in mouse liver using Northern blot analysis, suggesting that this receptor has an important function in maintaining expression of BACAT. A HNF-4α binding site was indeed demonstrated in the promotor of the BACAT gene [33]. These animals showed an altered bile acid pattern with increased unconjugated bile acids in serum. They also showed an increase in glycine-conjugated bile acids. It has been shown that the mouse BACAT does not contain glycine conjugating activity [25], therefore this suggested that there may be a further unidentified BACAT gene which is activated in the HNF-4α-null mouse model, and which can conjugate bile acids to glycine.

Identification of a putative new peroxisomal BACAT

BACAT shows approximately 40-45% sequence identity to a family of enzymes known as acyl-CoA thioesterases. These enzymes have been identified in several cellular compartments such as cytosol, mitochondria and peroxisomes (for review see [22]. Database searches using both BACAT and acyl-CoA thioesterase sequences, identified two further genes in mouse which are related to both BACAT and acyl-CoA thioesterases. The products of these two genes show approximately 55% sequence identity to BACAT and are expressed in liver, kidney, gallbladder and weakly in proximal and distal intestine. Both these enzymes contain the classical peroxisomal type I targeting signal of –SKL at their carboxyterminal ends, which targets the proteins to peroxisomes. We therefore suggest that one of these enzymes could be the new candidate peroxisomal BACAT. We are currently expressing these gene products as recombinant proteins to examine if they have bile acid conjugating activity. However, we have encountered problems to obtain soluble protein in E. coli, but are currently working on other expression systems.

In conclusion, we have provided evidence for a novel peroxisomal BACAT, which may conjugate de-novo synthesized bile acids in peroxisomes.

Figure legends

Fig. 1: Differential regulation of peroxisomal and cytosolic BACAT activity and protein following treatment with WY-14,643. (A) Cytosol and purified peroxiomes were isolated from pooled livers from untreated (black bars) and WY-14,643 treated (grey bars) mice. BACAT activity was measured in each fraction as described in Experimental Procedures. Fig. adapted from [24]. **(B)** Western blot analysis of protein isolated from cytosol (38 μg) or purified peroxisomes (5 μg) from control (untreated) or WY-14,643 treated mouse liver, using an affinity purified antibody to mouse BACAT.

Fig. 2: Mouse BACAT is localized in cytosol. The previously cloned mouse BACAT [25] was expressed as a green fluorescent fusion protein (with the C-terminal sequence –SQL) and the same plasmid mutated in the C-terminal to –SKL. Human skin fibroblasts were transfected with these BACAT-GFP plasmids and the cells were incubated with a rabbit GFP antibody followed by a CY3 conjugated affinity purified donkey anti-rabbit IgG and examined by immunofluorescence microscopy. **A;** mouse BACAT (-SQL), **B;** mouse BACAT-SKL mutant.

Fig. 3: The newly identified peroxisomal BACAT is localized in peroxisomes. The open reading frame of the putative peroxisomal BACAT was cloned as a green fluorescent fusion protein and human skin fibroblasts from a healthy control and a Zellweger patient were transfected with this plasmid. The cells were incubated with a rabbit GFP antibody followed by a CY3 conjugated affinity purified donkey anti-rabbit IgG and examined by immunofluorescence microscopy. (A) Control fibroblasts (B) Zellweger fibroblasts.

Fig. 4: Tissue expression of peroxisomal BACAT. Northern blot analysis was carried out on 20 μg of total RNA from SV/129 male mouse tissues. The filter was incubated with a ^{32}P-labelled mouse peroxisomal BACAT cDNA probe as described in Experimental Procedures. BAT: brown adipose tissue, WAT: white adipose tissue.

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Fig. 1

A

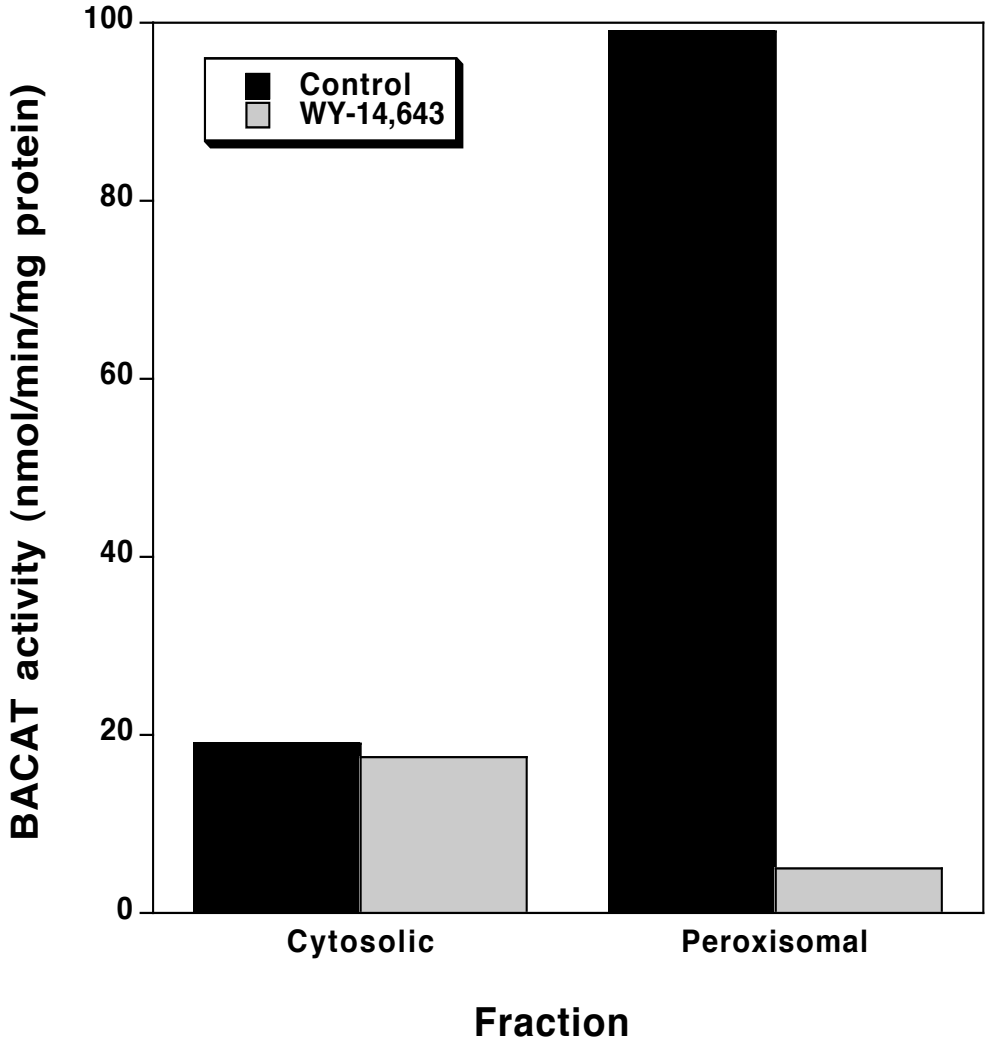
100
80
60
40
20
0
BACAT activity (nmol/min/mg protein)
Control
WY-14,643
Cytosolic
Peroxisomal
Fraction

Fig. 1

B

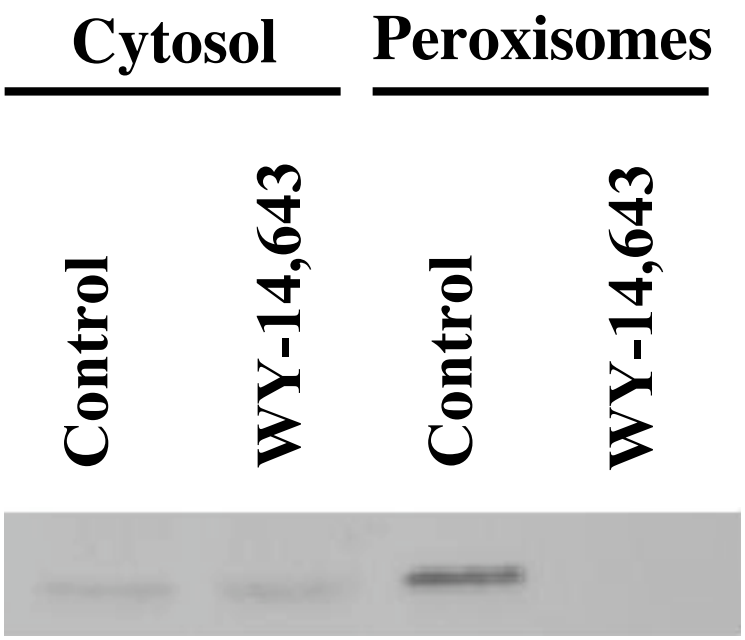

Fig. 2

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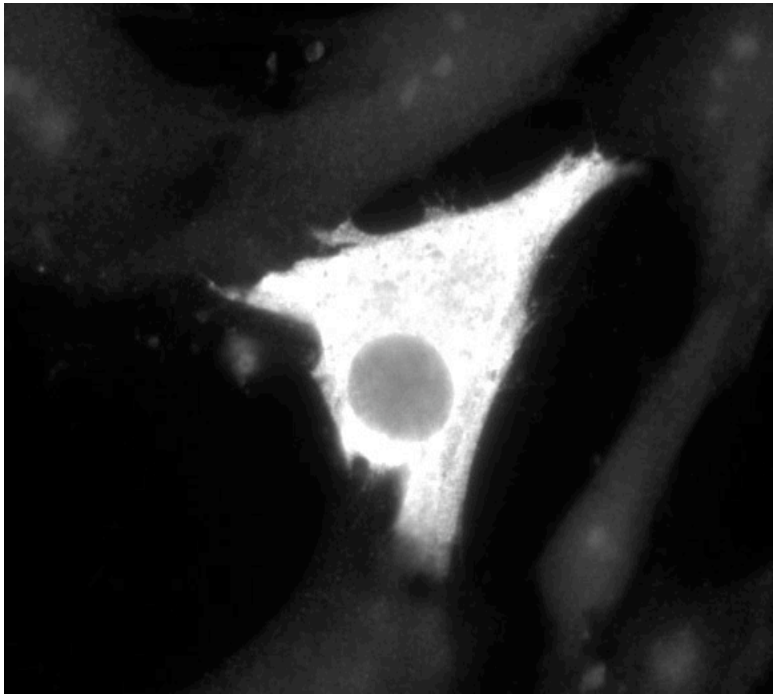

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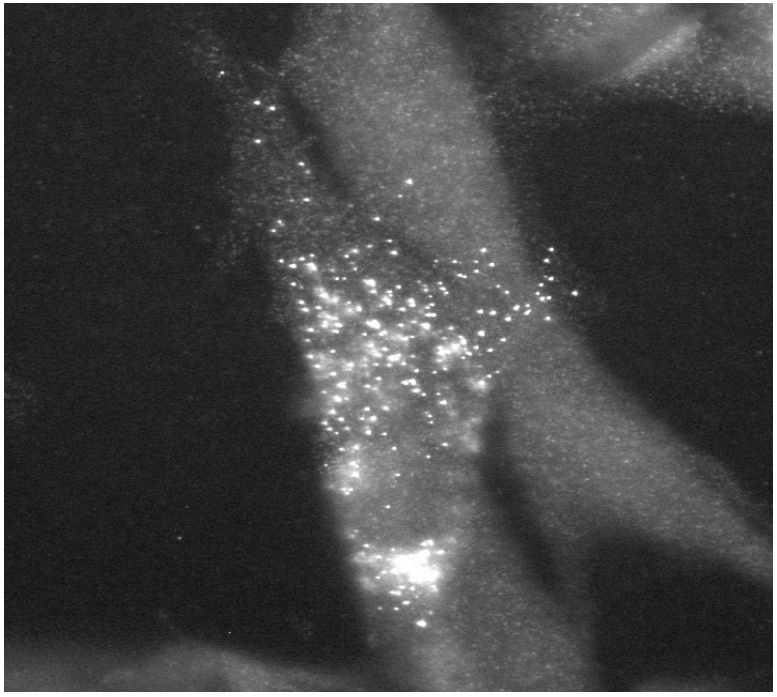
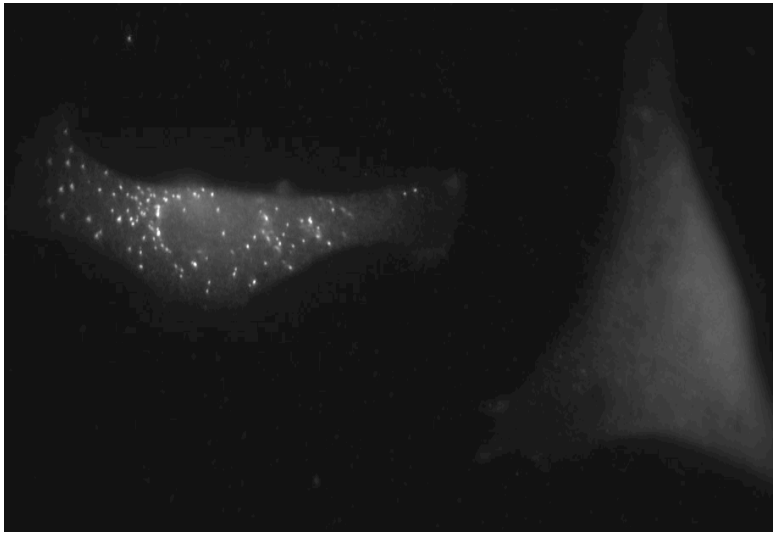


Fig. 3

A



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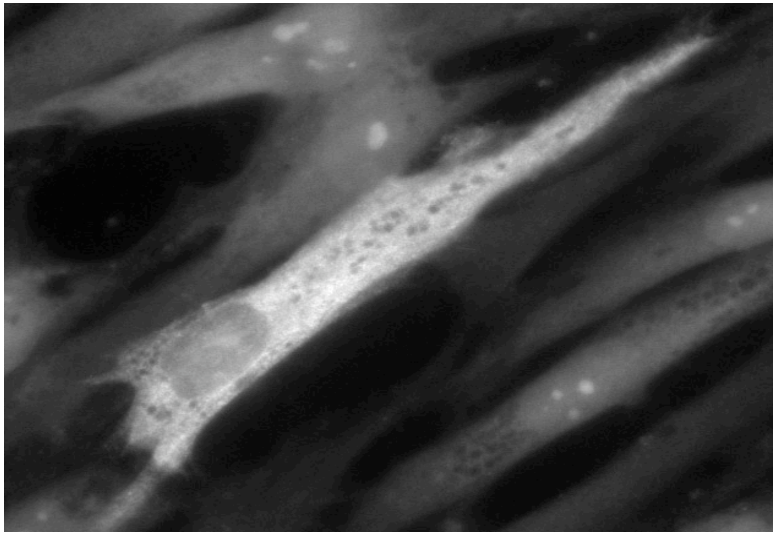
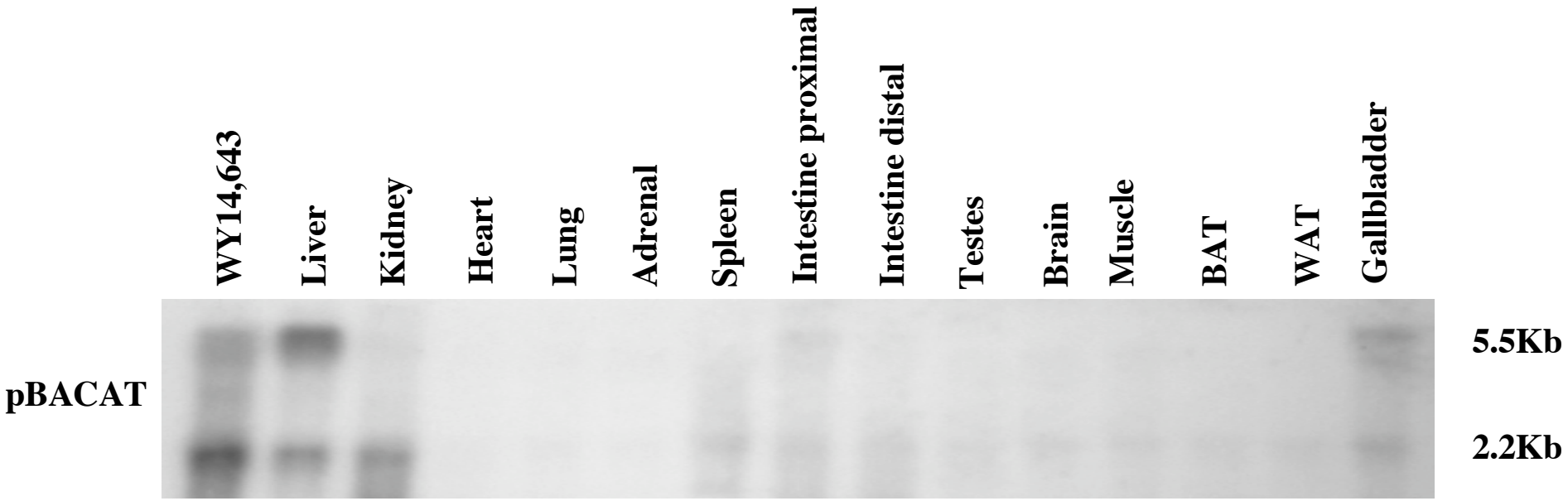


Fig. 4


WY14,643
Liver
Kidney
Heart
Lung
Adrenal
Spleen
Intestine proximal
Intestine distal
Testes
Brain
Muscle
BAT
WAT
Gallbladder
pBACAT
5.5Kb
2.2Kb