Two Human ACAT2 mRNA Variants Produced by Alternative Splicing and Coding for Novel Isoenzymes

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Abstract Acyl coenzyme A:cholesterol acyltransferase 2 (ACAT2) plays an important role in cholesterol absorption. Human ACAT2 is highly expressed in small intestine and fetal liver, but its expression is greatly diminished in adult liver. The full-length human ACAT2 mRNA encodes a protein, designated ACAT2a, with 522 amino acids. We have previously reported the organization of the human ACAT2 gene and the differentiation-dependent promoter activity in intestinal Caco-2 cells. In the current work, two human ACAT2 mRNA variants produced by alternative splicing are cloned and predicted to encode two novel ACAT2 isoforms, named ACAT2b and ACAT2c, with 502 and 379 amino acids, respectively. These mRNA variants differ from ACAT2a mRNA by lack of the exon 4 (ACAT2b mRNA) and exons 4-5 plus 8-9-10 (ACAT2c mRNA). Significantly, comparable amounts of the alternatively spliced ACAT2 mRNA variants were detected by RT-PCR, and Western blot analysis confirmed the presence of their corresponding proteins in human liver and intestine cells. Furthermore, phosphorylation and enzymatic activity analyses demonstrated that the novel isoenzymes ACAT2b and ACAT2c lacked the phosphorylatable site SLLD, and their enzymatic activities reduced to 25%–35% of that of ACAT2a. These evidences indicate that alternative splicing produces two human ACAT2 mRNA variants that encode the novel ACAT2 isoenzymes. Our findings might help to understand the regulation of the ACAT2 gene expression under certain physiological and pathological conditions.

Key words ACAT2 gene; alternative splicing; ACAT2 mRNA variant; isoenzyme

Acyl coenzyme A:cholesterol acyltransferase (ACAT) is a membrane-bound enzyme present in eukaryotic cells. It catalyzes the formation of cholesterol ester from long-chain fatty acyl coenzyme A and cholesterol [1], and participates in various physiological processes including cellular cholesterol homeostasis in tissue cells, dietary cholesterol absorption in small intestine, apoB-containing lipoprotein assembly in small intestine and liver, and steroidgenesis in the adrenal glands [2–4]. ACAT is also involved in the pathophysiological processes that lead to various human diseases, such as atherosclerosis [5–7] and Alzheimer's disease [8,9]. The ACAT inhibitor can decrease foam cell formation and amyloid β-peptide production [8,10]. For these reasons, ACAT has been one of the major pharmaceutical targets for developing cholesterol-lowering and anti-atherosclerosis agents [5–9].

So far, two ACAT (ACAT1 and ACAT2) genes have been characterized in eukaryotes. Chang *et al.* first reported human ACAT1 cDNA in 1993 [11]. According to the knock-out experiments and the conserved sequences of the ACAT1 gene, ACAT2 cDNA was identified in monkey, mouse or human by different laboratory [12–14]. The organizations of their genomic DNAs were then investigated [15–17]. In humans, ACAT1 is ubiquitously expressed in various tissues and cells, whereas ACAT2 is highly expressed in

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small intestine and fetal liver but is greatly diminished in adult liver [18–21]. In differentiating intestinal Caco-2 cells, ACAT2 protein content increases by 5- to 10-fold, but ACAT1 protein content remains relatively constant [20]. So far, it has been reported that the expression of human ACAT1 gene is regulated at different levels [22–24]. We have reported that human ACAT2 gene promoter displays cell-type specific and differentiation-dependent activity in intestinal Caco-2 cells [16]. To provide the molecular basis for regulation and function of the ACAT2 gene expression, here we report that alternative splicing produces the two human ACAT2 mRNA variants that encode two novel ACAT2 isoenzymes without phosphorylatable site SLLD and with lower enzymatic activity.

Materials and Methods

Reagents

All the restriction endonucleases, pGEM-T easy vector and moloney murine leukemia virus (MMLV) reverse transcriptase were ordered from Promega (Madison, USA). The expression vector pcDNA3, phenylmethylsulfonyl fluoride (PMSF), Trizol total RNA extraction kit and cell culture reagents were purchased from Invitrogen (Carlsbad, USA). Anti-ACAT2 antibodies DM54, described previously, and A21, newly prepared, were used for Western blot analysis [25]. Goat antirabbit IgG conjugated with horseradish peroxidase (HRP) was from Pierce (Rockford, USA). Anti-β-actin antibody and enhanced chemiluminescence (ECL) detection reagent were from Santa Cruz Biotechnology (Santa Cruz, USA). Protease inhibitor cocktail was from Sigma (St. Louis, USA). *Taq* DNA polymerase and dNTPs were from Sino-American Biotech (Shanghai, China). Dithiothreitol (DTT) was from Amersham Biosciences (Piscataway, USA). GSH-Sepharose 4B and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham Pharmacia Biotech (Uppsala, Sweden). All the oligonucleotides were synthesized with an automated DNA synthesizer in the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. [^{3}H]oleoyl-coenzyme A was synthesized as described previously [26].

Cell culture and human tissues

All the cell lines used were maintained in basal medium supplemented with fetal bovine serum (FBS) and antibiotics in a 37 ºC incubator with 5% CO_2 and 95% air. The human colon carcinoma cell line Caco-2 was maintained in Dulbecco's modified Eagle's medium containing 20% FBS, 100 U/ml of penicillin and 100 U/ml of streptomycin; cells seeded for 4, 6, 8 or 12 d were designated as Caco-2 cell differentiation for 0, 2, 4 or 8 d (0, 2, 4 or 8 d post-confluency), respectively. Human hepatoblastoma cell line HepG2 and cervical cancer cell line HeLa were maintained in the same medium as Caco-2, except that it also contained 10% FBS. Human acute monocytic leukemia cell line THP-1 was maintained in RPMI 1640 medium containing 10% FBS. Human embryonal kidney cell line HEK293 and Chinese hamster ovary cell line AC29 were grown in minimal essential medium and Ham's F12 medium containing 10% FBS, respectively. The human tissues were obtained from national hospitals (Shanghai, China). Consents for removal of tissues for research purposes were obtained from donors or their relatives. The tissues were rapidly frozen and stored in liquid nitrogen until use.

Reverse transcription-polymerase chain reaction (RT-PCR)

The 4 μg of total RNA prepared from cultured cells or tissues by the single step acid guanidinium thiocyanate phenol chloroform method (Invitrogen) was annealed with 0.5 μg of oligo(dT_{18}) in a total volume of 20 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT. The single-stranded cDNAs (ss-cDNAs) were synthesized by adding 1 μl (200 U) MMLV reverse transcriptase and 1 μl of dNTPs (10 mM of each of dATP, dCTP, dGTP and dTTP) to the annealed products and incubating at 37 °C for 60 min. The 1 μl of ss-cDNA was added to a PCR mixture in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0. 2 mM of each of dATP, dCTP, dGTP and dTTP, 0.4 μM of each set of the forward/reverse primers, and 2.5 U of *Taq* DNA polymerase. The control experiments were performed to assure that the PCR product was amplified within the linear amplification range. PCR products (10 μl) were separated and stained on agarose gel, and quantified using UVP Labwork (Version 4.0) software. The sequences of forward and reverse primers used in PCR to amplify the different fragments of human ACAT2 cDNA are listed in **Table 1**. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment (291 bp) was amplified as the control using the forward/reverse primer set GAPDHF (5′-ACCACAGTCCATGCCATCAC-3′)/GAPDHR (5′-TCCACCACCCTGTTGCTGTA-3′).

Plasmids

The whole coding sequences of human ACAT2 cDNA

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Table 1 Forward (F) and reverse (R) primers used in RT-PCR to amplify the different fragments of human ACAT2 cDNA

Primer	Sequence (5′→3′)	Corresponding location
C41 (F)	GGAGACCGCACCATGGAG	77–94 (exon 1)
C31 (F)	TGATCTTCAGCTTCGGACAG	555–574 (exon 6)
C14 (R)	GCTGAAGATCAGTAGGTCAAACTCC	565–541 (exon 6)
C21 (R)	GGAAGAGTATCAGCATGACG	1448–1429 (exon 13)
C42 (R)	GACCTCTAGGTATGGCAGGAC	1662–1642 (exon 15)

The primer locations are according to the ACAT2 sequence (GenBank accession No. AH010689) that shows three transcription start sites, designated 1, 17 and 34. The start and stop codons are underlined in the primers C41 (F) and C42 (R).

fragments were amplified by RT-PCR using the forward/reverse primer set C41/C42, cloned into pGEM-T easy vector, and identified by digestion of the restriction enzyme *Eco*RI and DNA sequencing. The human ACAT2 cDNA fragments amplified from the identified plasmids pGEM-ACAT2a, pGEM-ACAT2b and pGEM-ACAT2c were digested with *Eco*RI and *Xba*I, then inserted into the *Eco*RI/*Xba*I sites of the vector pcDNA3 to generate three expression plasmids pACAT2a, pACAT2b and pACAT2c.

Transfection

The human ACAT2 expression plasmids were individually transfected into AC29 cells using the calcium phosphate co-precipitation method [27]. Briefly, cells were cultured in 60 mm dishes at a density of 5×10^5 cells per 5 ml of culture medium containing 10% FBS for 24 h, and fed with fresh medium for another 2 h before transfection. The 1.2 ml of transfection mixture (containing 12 μg DNA) for a 60 mm dish was prepared by gently mixing equal volumes of DNA (125 mM calcium ion at final concentration) and HeBS (21 mM HEPES, pH 7.1, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM glucose at final concentration), added into the medium and incubated with the cells at 37 ºC for 9 h. The transfected cells were rinsed twice with phosphate buffered saline (PBS) and grown for 48 h in culture medium for Western blot analysis and ACAT activity assay.

Western blot

The cell lysates from the cultured cells were prepared by harvesting with a lysis buffer containing 10% SDS, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM DTT, and 1% (*V*/*V*) protease inhibitor cocktail, incubating at 37 ºC for 20 min, then shearing with a syringe fitted with an 18-G needle to homogeneity. Protein concentrations of the cell lysates were determined by a modified Lowry method [28]. The protein samples were then subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After gel separation, the proteins were transferred to a nitrocellulose membrane. The transferred membrane was treated successively at room temperature with the following solutions: 5% milk in TBST (50 mM Tris-HCl, pH 7.6, 0.15 M NaCl, and 0.05% Tween-20) for 2 h, the affinity-purified rabbit anti-ACAT2 IgGs or anti-β-actin antibodies (control) for 3 h, and HRP-conjugated goat anti-rabbit antibodies for an additional 1 h. After incubation, the membrane was washed extensively with TBST, then with TBS (50 mM Tris-HCl, pH 7.6, and 0.15 M NaCl). The immunoreactive bands were visualized using ECL detection reagent and developed with Hyperfilm-ECL (Amersham Pharmacia Biotech).

In vitro phosphorylation assay

The *in vitro* phosphorylation assay was performed as described previously with slight modifications [29]. Briefly, AC29 cells were cultured in a 60 mm dish, washed twice with PBS in an ice-bath, then incubated with 350 μl of a lysis buffer (10 mM Tris-HCl, pH 7.4, 1.0% Triton X-100, 0.5% NP-40, 150 mM NaCl, 1.0 mM EDTA, and 0.2 mM PMSF) at 4 ºC for 30 min. The cell lysates were harvested, sheared with a syringe fitted with a 26-G needle to homogeneity, and centrifuged at 16,000 *g* at 4 ºC for 30 min. The supernatant obtained was mixed with an equal volume of dilution buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2 mM PMSF, 20 mM $MgCl_2$ and 1 mM DTT) as a casein kinase II (CK2) mixture. The fusion proteins GST-ACAT2aN and GST-ACAT2bN were expressed in *Escherichia coli* harboring the GST (227 amino acids) fusion expression plasmids and purified with the GSH-Sepharose 4B column (Amersham Pharmacia Biotech) as described previously [30]. The phosphorylation reactions were performed by adding the purified fusion protein (2 μg GST-ACAT2aN or 6 μg GST-ACAT2aN), [γ-^{32}P]-ATP (50 μCi) and cold ATP (final concentration 25 μM) to 200 μl of CK2 mixture, and incubating at 37 ºC for 15 min. The 20 μl of GSH-Sepharose 4B beads were added to the

phosphorylated reaction mixture and incubated at 4 ºC for 1 h. The precipitate was harvested by centrifugation and washed four times using PBS. The pellets were run on SDS-PAGE, and then the gel was subsequently stained with Coomassie brilliant blue and autoradiographed with the PhosphorImager scanning system (Molecular Dynamics, Sunnyvale, USA).

ACAT activity assay

The ACAT activity assay using the *in vitro* reconstitution method was performed as described previously [25].

Results

Multiple human ACAT2 mRNAs detected by RT-PCR

We have reported the organization of human ACAT2 genomic DNA and cloned its promoter [16]. It was also noticed that the different-sized fragments of human ACAT2 cDNA were amplified using total RNA from intestinal Caco-2 cells by RT-PCR (data not shown), suggesting that there might be more than one kind of human ACAT2 mRNA produced in the tissue cells. To address this possibility, RT-PCR using various primers [**Table 1** and **Fig. 1(A)**] was performed with the total RNAs prepared from human cell lines and tissues. As shown in **Fig. 1(B)**, using a forward/reverse primer set C31/C21, two cDNA fragments, one with the predicted 894 bp and another smaller (arrowed), were respectively amplified from the RT products of HepG2, Caco-2, fetal lung, fetal liver and cancer liver; only one bigger band (894 bp) with very low amount was observed from that of THP-1; and there are no detectable from those of HEK293, HeLa, adult liver and adjacent benign liver matched with the cancer liver. Significantly, comparable amounts of the two kinds of ACAT2 mRNA were detected in the fetal tissues and cancer liver. Further experiments using the other forward/reverse primer set C41/C14 or C41/C21 for amplification consistently revealed the fragments with the predicted size (489-bp or 1372-bp) and smaller size (arrowed) [**Fig. 1(C)**, lanes 2, 4 or 7, 8],

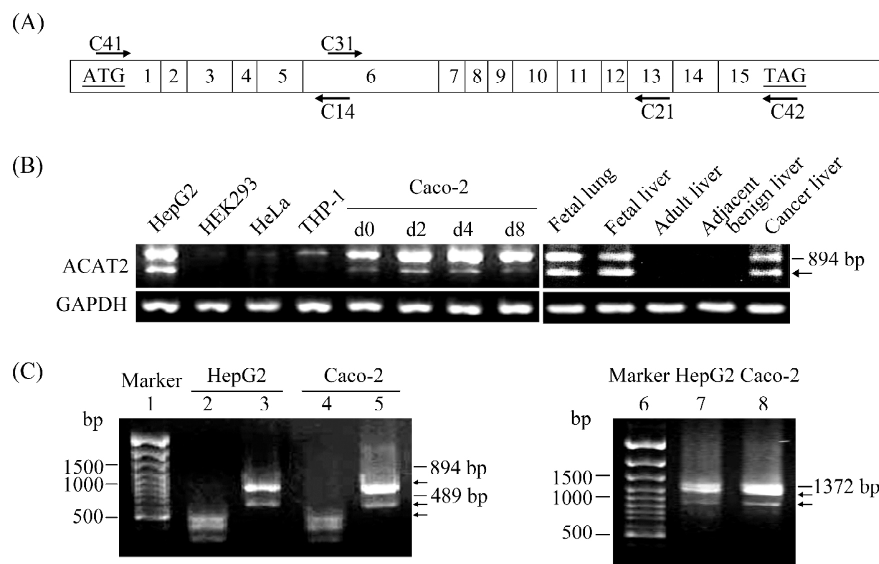

Fig. 1 RT-PCR analysis

Total RNA samples were prepared from human cells and tissues. The ACAT2 and GAPDH mRNAs were estimated semi-quantitatively by RT-PCR with different sets of primers. The details of the experiments are described in "Materials and Methods". (A) Schematic diagram illustrating the locations of the PCR primers specific for human ACAT2 cDNA. The arrows indicate the orientations of forward (C41 and C31) and reverse (C42, C21 and C14) primers. The numbers of exons and the underlined start and stop codons of human ACAT2 cDNA are shown. (B) The primer sets (C31/C21 and GAPDHF/GAPDHR) and total RNAs from the different human cell lines and tissues, as indicated at the top, were used to amplify the fragments of human ACAT2 and control GAPDH cDNAs. The total RNA samples from intestinal Caco-2 cells differentiated for 0, 2, 4 or 8 d were designed as d0, d2, d4 or d8. The arrow indicates the amplified smaller fragment of human ACAT2 cDNA. (C) Using the primer set C31/C21 (lanes 3 and 5) as the control, the other primer sets C41/C14 (lanes 2 and 4) and C41/C21 (lanes 7 and 8) were used to amplify different fragments of human ACAT2 cDNAs from the HepG2 and Caco-2 (differentiated for 4 d) total RNAs. The arrows indicate the amplified smaller fragments of human ACAT2 cDNA per primer set. Lanes 1 and 6, 100-bp ladder marker.

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confirming that multiple ACAT2 mRNAs were produced in human cells.

Human ACAT2 mRNA variants matured by alternative splicing

Next, the forward (C41) and reverse (C42) primers, which are matched with the sequences in the 1st and 15th exons [**Fig. 1(A)**], were used to amplify the whole coding sequence of human ACAT2 cDNA. The PCR products were cloned into pGEM-T easy vector. The inserted plasmids were identified by digestion with the restriction enzymes and DNA sequencing. Three different-sized fragments (arrowed) were cloned, as shown in **Fig. 2(A)**. DNA sequencing of these three cloned fragments demonstrated that three distinct human mRNA species containing different exons [**Fig. 2(B)**, right] were matured from the same ACAT2 pre-mRNA [**Fig. 2(B)**, left] by comparing with the published sequences of human ACAT2 genomic and complementary DNAs. The bigger one (designated ACAT2a mRNA) is the same as the ACAT2 cDNA reported previously with all 15 exons [16,17]; the other smaller two (named ACAT2b and ACAT2c mRNAs) lack the exon 4 (60 nt) and the exons 4-5 plus 8-9-10 (429 nt), respectively. ACAT2a and ACAT2b mRNAs are very similar in size, so they are sometimes observed as one band in agarose gel electrophoresis. These data clearly show that alternative splicing produces human ACAT2 mRNA variants.

Two novel isoforms encoded by human ACAT2 mRNA variants

The deduced amino acid sequences of human ACAT2a, ACAT2b and ACAT2c from the alternatively spliced mRNA variants are shown in **Fig. 3**. The full-length human ACAT2a is a 522 amino acid protein, as described [14,16,17], and the two novel isoforms ACAT2b and ACAT2c have 502 and 379 amino acids in which the absences of 20 and 143 amino acids are led by the in-frame deficiencies of the

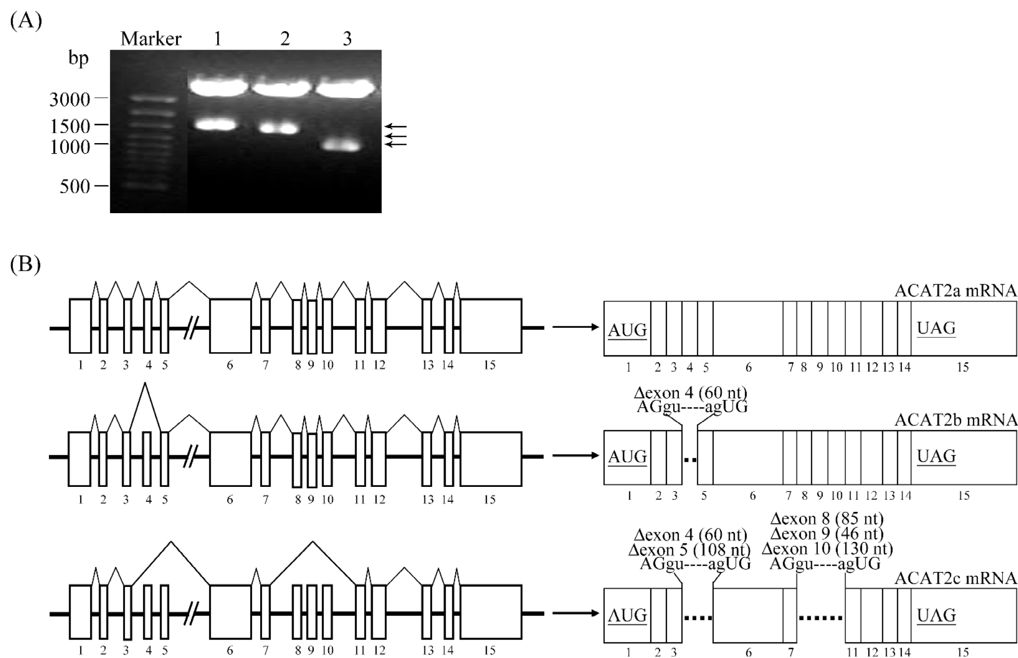

Fig. 2 Alternative splicing in maturation of human ACAT2 mRNA variants

(A) Human ACAT2 cDNA fragments cloned from the RT-PCR products. The products from PCR using the forward/reverse primer set C41/C42 were cloned into pGEM-T easy vector. The plasmids prepared from the positive colonies were digested with the restriction endonuclease *Eco*RI and running agarose gel. Three different-sized fragments inserted into the plasmids pGEM-ACAT2a, pGEM-ACAT2b and pGEM-ACAT2c, prepared from different bacterial colonies (indicated by numbers 1, 2 and 3 at the top), are arrowed. (B) Schematic diagram showing the maturation of human ACAT2 mRNA variants. The sequences of the three cloned fragments of human ACAT2 cDNA described above were determined by DNA sequencing, and used to generate the maturations of mRNA variants (right) from the same pre-mRNA (left). The two new variants (middle and bottom) were designed ACAT2b and ACAT2c mRNAs, respectively, and the previously reported one (top) was named ACAT2a mRNA. The numbers of exons and the underlined start and stop codons of human ACAT2 mRNA are shown.

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ACAT2a   MEPGGARLRL QRTEGLGGER EPQPCGDGNT ETHRAPDLVQ WTRHMEAVKA QLLEQAQGQL   60
ACAT2b   MEPGGARLRL QRTEGLGGER EPQPCGDGNT ETHRAPDLVQ WTRHMEAVKA QLLEQAQGQL   60
ACAT2c   MEPGGARLRL QRTEGLGGER EPQPCGDGNT ETHRAPDLVQ WTRHMEAVKA QLLEQAQGQL   60

ACAT2a   RELLDRAMRE AIQSYPSQDK PLPPPPPGSL SRTQEPSLGK QKVFIIRKSL LDELMEVQHF  120
ACAT2b   RELLDRAMRE AIQSYPSQDK PLPPPPPGSL SR-------- ---------- --ELMEVQHF  100
ACAT2c   RELLDRAMRE AIQSYPSQDK PLPPPPPGSL SR-------- ---------- ----------   92

ACAT2a   RTIYHMFIAG LCVFIISTLA IDFIDEGRLL LEFDLLIFSF GQLPLALVTW VPMFLSTLLA  180
ACAT2b   RTIYHMFIAG LCVFIISTLA IDFIDEGRLL LEFDLLIFSF GQLPLALVTW VPMFLSTLLA  160
ACAT2c   ---------- ---------- --------LL LEFDLLIFSF GQLPLALVTW VPMFLSTLLA  124

ACAT2a   PYQALRLWAR GTWTQATGLG CALLAAHAVV LCALPVHVAV EHQLPPASRC VLVFEQGRFL  240
ACAT2b   PYQALRLWAR GTWTQATGLG CALLAAHAVV LCALPVHVAV EHQLPPASRC VLVFEQGRFL  220
ACAT2c   PYQALRLWAR GTWTQATGLG CALLAAHAVV LCALPVHVAV EHQLPPASRC VLVFEQGRFL  184

ACAT2a   MKSYSFLREA VPGTLRARRG EGIQAPSFSS YLYFLFCPTL IYRETYPRTP YVRWNYVAKN  300
ACAT2b   MKSYSFLREA VPGTLRARRG EGIQAPSFSS YLYFLFCPTL IYRETYPRTP YVRWNYVAKN  280
ACAT2c   MKSYSFLREA VPGTLRARRG ---------- ---------- ---------- ----------  204

ACAT2a   FAQALGCVLY ACFILGRLCV PVFANMSREP FSTRALVLSI LHATLPGIFM LLLIFFAFLH  360
ACAT2b   FAQALGCVLY ACFILGRLCV PVFANMSREP FSTRALVLSI LHATLPGIFM LLLIFFAFLH  340
ACAT2c                                                    IFM LLLIFFAFLH  217

ACAT2a   CWLNAFAEML RFGDRMFYRD WWNSTSFSNY YRTWNVVVHD WLYSYVYQDG LRLLGARARG  420
ACAT2b   CWLNAFAEML RFGDRMFYRD WWNSTSFSNY YRTWNVVVHD WLYSYVYQDG LRLLGARARG  400
ACAT2c   CWLNAFAEML RFGDRMFYRD WWNSTSFSNY YRTWNVVVHD WLYSYVYQDG LRLLGARARG  277

ACAT2a   VAMLGVFLVS AVAHEYIFCF VLGFFYPVML ILFLVIGGML NFMMHDQRTG PAWNVLMWTM  480
ACAT2b   VAMLGVFLVS AVAHEYIFCF VLGFFYPVML ILFLVIGGML NFMMHDQRTG PAWNVLMWTM  460
ACAT2c   VAMLGVFLVS AVAHEYIFCF VLGFFYPVML ILFLVIGGML NFMMHDQRTG PAWNVLMWTM  337

ACAT2a   LFLGQGIQVS LYCQEWYARR HCPLPQATFW GLVTPRSWSC HT                     522
ACAT2b   LFLGQGIQVS LYCQEWYARR HCPLPQATFW GLVTPRSWSC HT                     502
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Fig. 3 Alignment of amino acid sequences of isoforms encoded by human ACAT2 mRNA variants

The predicted amino acid sequences of ACAT2a, ACAT2b and ACAT2c are numbered, with the initiator methionine denoted as 1. Three gaps, one for ACAT2b and two for ACAT2c, are indicated by the broken lines.

exon 4 (60-nt) of ACAT2b mRNA and the exons 4-5 plus 8-9-10 (429-nt) of ACAT2c mRNA, respectively. The expression plasmids for human ACAT2 isoforms were then constructed by inserting the cloned human ACAT2 cDNAs into the pcDNA3 vector [**Fig. 4(A)**] and transiently transfected into the mutant Chinese hamster ovary cell line AC29 without endogenous ACAT. Western blot analysis with β-actin as the control showed that the transfected AC29 cells highly expressed human ACAT2a, ACAT2b and ACAT2c with different molecular weights [**Fig. 4(B)**], indicating that the alternatively spliced human ACAT2 mRNA variants could encode two novel isoforms ACAT2b and ACAT2c in the cells besides the reported full-length human ACAT2a. The endogenous expression of human ACAT2 isoforms in HepG2 and Caco-2 cells was also observed by Western blot [**Fig. 4(C)**]. But ACAT2a and ACAT2b were so similar in size that they might form one band in SDS-PAGE. Also, the human ACAT2 isoforms were not detected in HEK293, HeLa and THP-1 cells, similar to the results of RT-PCR [**Fig. 1(B)**].

Two novel isoenzymes without phosphorylatable site SLLD and with lower enzymatic activity

A comparison between the N-terminal amino acid sequences of human ACAT2 isoforms showed that the two novel identified isoforms ACAT2b and ACAT2c lacked a potential CK2 phosphorylation site SLLD located at the 20 amino acid region (**Fig. 3**, underlined) encoded by exon 4 which was in-frame deficiency in both ACAT2b and ACAT2c mRNAs. This phosphorylation site SLLD for the CK2 has been found at N-terminal regions of all the reported ACAT2 and ACAT1 species [**Fig. 5(A)**], implying that it

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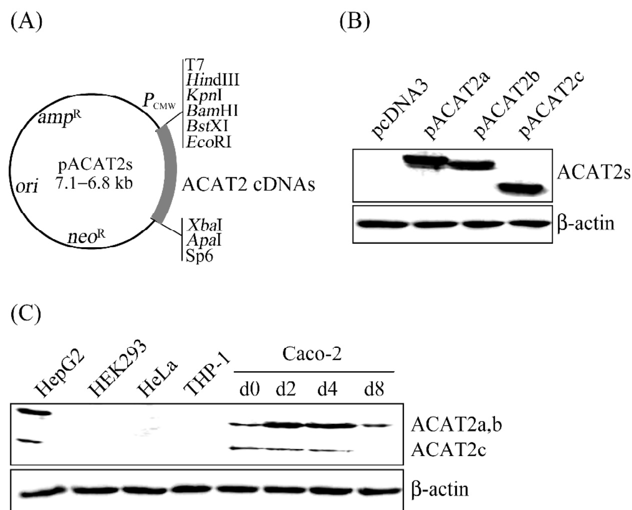

Fig. 4 Western blot analysis for human ACAT2 proteins from transiently transfected AC29 cells and various human cell lines
(A) Human ACAT2 expression plasmids constructed by insertion of the cloned cDNAs into the vector pcDNA3. (B,C) Western blot analysis. The protein samples were freshly prepared from AC29 cells transiently transfected with the human ACAT2 expression plasmids (pACAT2a, pACAT2b and pACAT2c) or the empty vector pcDNA3 (B), and various human cell lines (C). The protein samples prepared from intestinal Caco-2 cells differentiated for 0, 2, 4 or 8 d were designed as d0, d2, d4 or d8. The experimental performances are described in detail under "Materials and Methods". The data shown with β-actin as the control are representative of three separate experiments.

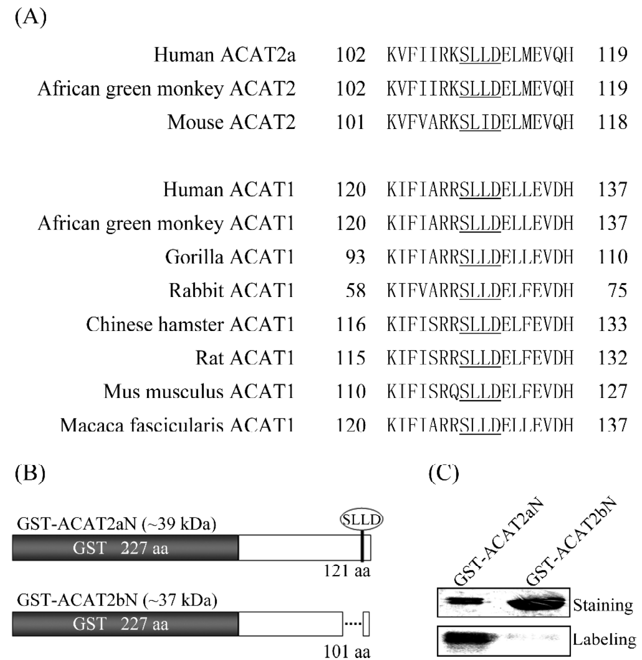

Fig. 5 *In vitro* phosphorylation assay of human ACAT2 protein
(A) Casein kinase II (CK2) phosphorylation site SLLD at the N-terminal region of various ACATs. Numbers representing the amino acid (aa) positions of ACAT1 and ACAT2 proteins are shown according to the GenBank accession No. AH010689 (human ACAT2a), AF053234 (African green monkey ACAT2), AF078751 (mouse ACAT2), NM003101 (human ACAT1), AF053336 (African green monkey ACAT1), AF354622 (gorilla ACAT1), U65393 (rabbit ACAT1), 60457 (Chinese hamaster ACAT1), O70536 (rat ACAT1), L42293 (mus musculus ACAT1), and AF053337 (macaca fascicularis ACAT1). The CK2 phosphorylation sites at the N-terminal region of various ACATs are underlined. (B) Schematic diagram showing the fusion proteins GST-ACAT2aN and GST-ACAT2bN. The carrier GST (227 amino acids) and the N-terminus of human ACAT2 (121 amino acids for ACAT2a and 101 amino acids for ACAT2b) are shown as black and white regions, respectively. The phosphorylation site SLLD at the human ACAT2a N-terminus is indicated. (C) Staining and labeling of the GST-ACAT2aN and GST-ACAT2bN fusion proteins. The experimental performances are described in detail under "Materials and Methods". The experiments were repeated twice with similar results. aa, amino acid.

might be phosphorylatable and have more important roles in the cells. For the phosphorylation assay, the fusion proteins GST-ACAT2aN and GST-ACAT2bN were expressed by fusion of GST with the N-terminal region of ACAT2a (121 amino acids) and ACAT2b (101 amino acids), respectively, as shown in **Fig. 5(B)**. The results demonstrated that GST-ACAT2aN could be highly phosphorylatable, but GST-ACAT2bN had no labeling signal even when a large amount of protein was used [**Fig. 5(C)**]. Furthermore, the enzymatic activities of human ACAT2 isoforms were analyzed by the transient transfection of human ACAT2 expression plasmids into AC29 cells. ACAT activity analysis using the methods of the reconstitution *in vitro* (**Fig. 6**) and [^{3}H]oleate pulse *in vivo* (data not shown) indicated that the enzymatic activities of the two novel ACAT2 isoenzymes were lower than that of the full-length ACAT2a, approximately 25%–35% of ACAT2a activity.

Discussion

In this study, the multiple human ACAT2 mRNAs in human liver and intestine cells were analyzed by RT-PCR, cloning and DNA sequencing. Two mRNA variants were found to be matured by alternative splicing, and to code for the novel isoenzymes ACAT2b and ACAT2c without phosphorylation and with lower enzymatic activity. These findings might help to understand the regulation and roles of the ACAT2 gene expression under certain physiological and pathological conditions.

With respect to cell type- or tissue-specific expression of human ACAT2 mRNAs, some human cell lines, such as HepG2, HEK293, HeLa, THP-1 and Caco-2 were used in

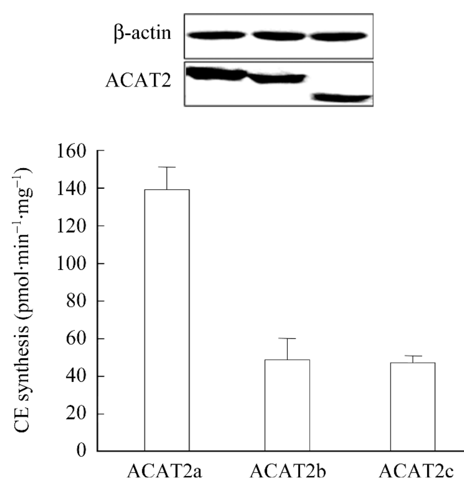

Fig. 6 Enzymatic activity analysis of human ACAT2 isoenzymes

AC29 cells were cultured at 5×10^5 cells per 5 ml in a 60 mm dish and transiently transfected with the human ACAT2 expression plasmids (pACAT2a, pACAT2b or pACAT2c). Western blot analysis with β-actin as the control for human ACAT2 isoenzymes was performed as described in "Materials and Methods". ACAT activities were assayed by the *in vitro* reconstitution method [25]. The ACAT activities in the bottom panel were normalized with their protein amounts from Western blot analysis in the top panel.

this study. The intestine cell line Caco-2 and liver cell line HepG2 expressed a high level of human ACAT2 mRNAs, but no or very low-level expression was observed in HEK293, HeLa and THP-1 cells. Comparable amounts of different kinds of ACAT2 mRNAs were significantly detected in the fetal tissues and the cancer liver [**Fig. 1(B)**]. The identification of multiple human ACAT2 mRNAs, which are at high levels in the fetal tissues (liver and lung) but very low levels in adult liver and the adjacent benign liver matched with the cancer liver [**Fig. 1(B)**], supports the premise that the ACAT2 gene is highly expressed in human fetal liver but greatly diminished in the human adult liver [20]. However, whether the mechanistic regulation of the multiple human ACAT2 mRNA expression takes place in a cell type- or tissue-specific manner remains unknown.

By comparing the sequences of the cloned human ACAT2 cDNA fragments with ACAT2 genomic DNA [16,17], it was found that alternative splicing matured ACAT2b and ACAT2c mRNA variants. The alternatively spliced mRNA variants differ from the ACAT2a mRNA by lack of the exon 4 (ACAT2b mRNA) and the exons 4-5 plus 8-9-10 (ACAT2c mRNA). Consistent results have been obtained with several sets of primers [**Fig. 1(B,C)**], therefore it is believed that the ACAT2 mRNA variants are authentic in human liver and intestine cells. The nucleotide sequences of the alternatively spliced mRNA variants lacking some coding regions does not cause the shifting of the open reading frame, implying that certain functional regulation might have existed during the ACAT2 mRNA maturation. It will be of interest to study whether the alternatively spliced ACAT2mRNA variants may be specific to some developmental stages, particular tissues or disease states.

Due to the in-frame deficiencies of exon 4 (60 nt) in ACAT2b mRNA and exons 4-5 plus 8-9-10 (429 nt) in ACAT2c mRNA [**Fig. 2(B)**], the two novel isoforms ACAT2b, with 502 amino acids, and ACAT2c, with 379 amino acids, are smaller than the full-length ACAT2a, with 522 amino acids (**Fig. 3**). Western blot analysis showed that the novel ACAT2 isoforms present in human liver and intestine cells [**Fig. 4(C)**], suggesting that they may fulfill common essential roles under certain physiological or pathological conditions. So far, the subunit structure and functional domains of the ACAT2 holoenzyme are not known. In this study, phosphorylation and enzymatic activity analyses demonstrated that the novel isoenzymes ACAT2b and ACAT2c lacked the phosphorylatable site SLLD (**Fig. 3**, underlined) and their enzymatic activities reduced to 25%–35% of ACAT2a activity (**Fig. 6**). The phosphorylatable site SLLD has been found at N-terminal regions of all the reported ACAT2 and ACAT1 species [**Fig. 5(A)**, underlined] and can be recognized by CK2, a ubiquitous protein kinase responsible for the phosphorylation of Ser and Thr residues at its recognition motif S/TXXD/E [31]. Thus, it is postulated that the phosphorylation and dephosphorylation of ACATs might be important for the regulation activity of these aligomeric and allosteric enzymes, and that the lower enzymatic activity of the novel ACAT2 isoenzymes lacking the phosphorylatable site SLLD could not be regulated as protection against certain stress conditions. In addition, human ACAT2c, with abundant amino acid deficiency, may cause a great change in the membrane, according to the published membrane topology of ACAT1 and ACAT2 [32–35]. Further investigation on regulation of ACAT activity for common essential roles will be conducted in the future.

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