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MOLECULAR CLONING AND NUCLEOTIDE SEQUENCE OF COMPLEMENTARY DNA FOR HUMAN HEPATIC CYTOSOLIC ACETOACETYL-COENZYME A THIOLASE

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Summary: Complementary DNA for human cytosolic acetoacetyl-CoA thiolase (CT) was cloned with the use of anti-[human CT] antibody. The human CT cDNA clone (HCT10) has a 1479-bp insert and a 1191-base open reading frame encoding 397 amino acid residues. Partial polypeptide sequences from purified human CT were present in the deduced sequence. *In vivo* expression analysis showed that HCT10 encoded potassium-ion non-activated acetoacetyl-CoA thiolase with no 3-ketooctanoyl-CoA thiolase activity, which is characteristic for CT. The deduced amino acid sequence has a 34-57 % homology with 4 other human thiolases and 4 acetoacetyl-CoA thiolases of microorganisms.

Cytosolic acetoacetyl-CoA thiolase (EC.2.3.1.9) (CT) is one of five thiolases present in mammalian cells (1-3). CT was purified from chicken liver in 1973 (4) and from rat liver in 1974 (5). Rat CT is homotetramer of the 44 kD subunit. Rat CT activity is rich in brain, liver, and adrenals but poor in heart and muscle, hence, is likely to be involved in the pathway of steroid biosynthesis, catalyzing the synthesis of cytoplasmic acetoacetyl-CoA for substrate conversion into 3-hydroxy-3-methylglutaryl-CoA (5).

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Abbreviations used in this paper: CT, cytosolic acetoacetyl-CoA thiolase; T1, mitochondrial 3-ketoacyl-CoA thiolase; T2, mitochondrial acetoacetyl-CoA thiolase; PT, peroxisomal 3-ketoacyl-CoA thiolase; TFP, mitochondrial trifunctional protein; Tcp-1, t-complex polypeptide-1.

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Another acetoacetyl-CoA specific thiolase (T2) is present in mitochondria and plays roles in ketone body and isoleucine catabolism. T2 deficiency, known as β-ketothiolase deficiency, is an inherited organic aciduria which is well-defined at clinical and molecular levels (6-15). The other 3 thiolases, i.e. mitochondrial 3-ketoacyl-CoA thiolase (T1), mitochondrial enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (TFP) and peroxisomal 3-ketoacyl-CoA thiolase (PT), have substrate specificity for the longer 3-ketoacyl-CoA and play roles in fatty acid β-oxidation in mitochondria or peroxisomes (1-3). Deficiencies of the thiolases, except for T1, have been reported (16-18) and human cDNAs for these thiolases except for CT have been cloned (10, 19-21).

Descriptions of a few patients with CT deficiency have been reported (16, 22). Severe mental retardation and hypotonus are characteristic but clinical symptoms and laboratory findings including urinary organic acids are not so specific. The enzymatic confirmation of CT deficiency is difficult using fibroblasts because activities of T2 and T1 interfere with the CT assay. It seems important to clarify the molecular basis of CT deficiency to elucidate the role of CT in mammalian cells. Immunochemical and DNA analysis may facilitate an accurate diagnosis of CT deficiency. We have found no report of development of a CT antibody or of molecular cloning of mammalian CT cDNA.

We cloned the human hepatic CT cDNA and use was made of an anti-[human CT] antibody which we developed.

MATERIALS AND METHODS

Development of anti-[human CT] antibody. We earlier purified CT from a human autopsied liver (8), using the same method as for rat liver, as described by Middleton (5). 0.2 mg of the purified enzyme was emulsified with Freund's complete adjuvant, and injected into the axillary regions of a rabbit. A booster of the same dose was given twice and blood samples were collected two weeks after the last booster injection. The antibody was partially purified by fractionation with ammonium sulfate and dialyzed against 0.15M NaCl containing 10 mM potassium phosphate, pH 7.5. Immunoblot analysis was done using the ProtoBlot AP system (Promega).

Amino acid sequencing. The purified CT was partially digested with several proteases. Polypeptide fragments were separated in SDS-PAGE and transferred to a PVDF membrane. Amino acid sequence analysis was made on an Applied Biosystem Model 477A pulsed liquid-phase sequencer and an on-line Model 120A phenylthiohydantoin analyzer with a regular cycle program and chemicals from the manufacturer.

cDNA library screening and sequencing. A human hepatic λ gt 11 cDNA library (Clontech) was screened using the anti-[human CT]antibody. Further screening was done by the plaque hybridization method using a labelled fragment from a positive clone (HCT 16). Fragments were subcloned into pTZ 18U (U.S. Biochemicals) and sequenced by the dideoxy-chain termination method with a modified T7 DNA polymerase (U.S. Biochemicals).

Computer analysis. Homology searches in SWISS-PROT protein data base, multiple sequence alignment and calculation of molecular mass were done using DNASIS Mac (Hitachi Software Engineering). Sequence alignment was modified slightly, by eye.

In vivo expression analysis. Full-length cDNA was subcloned into an expression vector, pCAGGS (23). T2 and CT cDNAs were transfected into SV40-transformed fibroblasts of GK03

(T2 deficient cell lines) as described (14), except for the use of Lipofection (Gibco BRL) instead of TransfectAce.

RESULTS AND DISCUSSION

Development of anti-[human CT]antiserum. With three injections of the purified enzyme into a rabbit, the anti-[human CT] antibody was acquired. In immunoblot analysis, it recognized human CT in 30 μg of fibroblast extracts and 10 ng purified CT although it cross-reacted faintly with T2 (data not shown). For antibody screening of CT cDNA, we used it with no further purification.

Isolation and characterization of human CT cDNA. Only one positive clone (HCT 16) was obtained from 1.6×10^5 plaques of a human liver cDNA library by screening with the antibody. HCT 8 included an insert of about 800-bp. Multiple sequence alignment of the deduced amino acid sequence encoded in HCT16 with those of 4 other human thiolases (10, 19-21) revealed that the deduced sequence shared high homology but was not identical with the carboxy-terminal amino acid sequences of the other thiolases. We hence regarded HCT16 as CT cDNA. 2.0×10^5 clones of the same library were screened with HCT 16 as a probe, and HCT 8 and HCT 10 were obtained. The HCT 10 insert was 1479-bp long and included a 1191-bp open reading frame encoding 397 amino acids (Fig. 1). Amino acid sequences determined from the purified enzyme were identified in the deduced sequence (Fig. 1, underlined sequences). Molecular mass of the deduced amino acid sequence was calculated to be 41293.44, a value in accord with that estimated by SDS-PAGE (data not shown). Alignment of the amino acid sequence with other thiolases showed that Met^1 in HCT 10 was located at a reasonable position as the initiator methionine (Fig. 2). HCT 8 had a truncated 3' non-coding region which may be produced with the use of an alternative polyadenylation signal at position 1257-1263 (Fig.1. boxed).

In vivo expression of cDNA. Table I shows the results of *in vivo* expression analysis. To reduce the intrinsic acetoacetyl-CoA thiolase activity, SV40-transformed T2 deficient cell lines were used, as described(14). When human T2 cDNA was transfected, acetoacetyl-CoA thiolase activity in the absence of potassium ion was elevated 1.6 fold over the intrinsic activity and the activity in the presence of the ion was 6.7 times higher than that in the absence of the ion, a characteristic feature of T2(1). On the other hand, in transfection of CT cDNA (HCT 10), acetoacetyl-CoA thiolase activity was elevated about 60 fold over the intrinsic activity, in both the presence and absence of the ion. Moreover, there was no elevation in 3-ketooctanoyl-CoA thiolase activity. These results show that HCT 10 is the cDNA for human CT and encodes a full-length CT polypeptide.

Amino acid sequence homology. Figure 2 shows amino acid sequence alignment of human CT with 4 other human thiolases (HT2, HT1, HTFP, and HPT), one Saccharomyces acetoacetyl-CoA thiolase, ST (24), and three bacterial acetoacetyl-CoA thiolases, ZT (25), AET (26), ET (27). The CT sequence showed the closest homology (57 %) to both ZT and AET, although a 34-57 % homology among these thiolases was observed.

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                                                                                -31         -21         -11          -1
                                                                                GGGGCAG CGCAGGGCAG ACGGCGGCAG GAGAAGCAAG
          9          18          27          36          45          54          63          72          81
ATG AAT GCA GGC TCA GAT CCT GTG GTC ATC GTC TCG GCG GCG CGG ACC ATC ATA GGT TCC TTC AAT GGT GCC TTA GCT GCT
Met Asn Ala Gly Ser Asp Pro Val Val Ile Val Ser Ala Ala Arg Thr Ile Ile Gly Ser Phe Asn Gly Ala Leu Ala Ala

         90          99         108         117         126         135         144         153         162
GTT CCT GTC CAG GAC CTG GGC TCC ACT GTC ATC AAA GAA GTC TTG AAG AGG GCC ACT GTG GCT CCG GAA GAT GTG TCT GAG
Val Pro Val Gln Asp Leu Gly Ser Thr Val Ile Lys Glu Val Leu Lys Arg Ala Thr Val Ala Pro Glu Asp Val Ser Glu

        171         180         189         198         207         216         225         234         243
GTC ATC TTT GGA CAT GTC TTG GCA GCA GGC TGT GGG CAG AAT CCT GTT AGA CAA GCC AGT GTG GGT GCA GGA ATT CCC TAC
Val Ile Phe Gly His Val Leu Ala Ala Gly Cys Gly Gln Asn Pro Val Arg Gln Ala Ser Val Gly Ala Gly Ile Pro Tyr

        252         261         270         279         288         297         306         315         324
TCT GTT CCA GCA TGG AGC TGC CAG ATG ATC TGT GGG TCA GGC CTA AAA GCT GTG TGC CTT GCA GTC CAG TCA ATA GGG ATA
Ser Val Pro Ala Trp Ser Cys Gln Met Ile Cys Gly Ser Gly Leu Lys Ala Val Cys Leu Ala Val Gln Ser Ile Gly Ile

        333         342         351         360         369         378         387         396         405
GGA GAC TCC AGC ATT GTG GTT GCA GGA GGC ATG GAA AAT ATG AGC AAG GCT CCT CAC TTG GCT TAC TTG AGA ACA GGA GTA
Gly Asp Ser Ser Ile Val Val Ala Gly Gly Met Glu Asn Met Ser Lys Ala Pro His Leu Ala Tyr Leu Arg Thr Gly Val

        414         423         432         441         450         459         468         477         486
AAG ATA GGT GAG ATG CCA CTG ACT GAC AGT ATA CTC TGT GAT GGT CTT ACA GAT GCA TTT CAC AAC TGT CAT ATG GGT ATT
Lys Ile Gly Glu Met Pro Leu Thr Asp Ser Ile Leu Cys Asp Gly Leu Thr Asp Ala Phe His Asn Cys His Met Gly Ile

        495         504         513         522         531         540         549         558         567
ACA GCT GAA AAT GTA GCC ACA AAA TGG CAA GTG AGT AGA GAA GAT CAG GAC AAG GTT GCA GTT CTG TCC CAG AAC AGG ACA
Thr Ala Glu Asn Val Ala Thr Lys Trp Gln Val Ser Arg Glu Asp Gln Asp Lys Val Ala Val Leu Ser Gln Asn Arg Thr

        576         585         594         603         612         621         630         639         648
GAG AAT GCA CAG AAA GCT GGC CAT TTT GAC AAA GAG ATT GTA CCA GTT TTG GTG TCA ACT AGA AAA GGT CTT ATT GAA GTT
Glu Asn Ala Gln Lys Ala Gly His Phe Asp Lys Glu Ile Val Pro Val Leu Val Ser Thr Arg Lys Gly Leu Ile Glu Val

        657         666         675         684         693         702         711         720         729
AAA ACA GAT GAG TTT CCT CGC CAT GGG AGC AAC ATA GAA GCC ATG TCC AAG CTA AAG CCT TAC TTT CTT ACT GAT GGA ACG
Lys Thr Asp Glu Phe Pro Arg His Gly Ser Asn Ile Glu Ala Met Ser Lys Leu Lys Pro Tyr Phe Leu Thr Asp Gly Thr

        738         747         756         765         774         783         792         801         810
GGA ACA GTC ACC CCA GCC AAT GCT TCA GGA ATA AAT GAT GGT GCT GCA GCT GTT GCT CTT ATG AAG AAG TCA GAA GCT GAT
Gly Thr Val Thr Pro Ala Asn Ala Ser Gly Ile Asn Asp Gly Ala Ala Ala Val Ala Leu Met Lys Lys Ser Glu Ala Asp

        819         828         837         846         855         864         873         882         891
AAA CGT GGG CTT ACA CCT TTA GCA CGG ATA GTT TCC TGG TCC CAA GTG GGT GTG GAG CCT TCC ATT ATG GGA ATA GGA CCA
Lys Arg Gly Leu Thr Pro Leu Ala Arg Ile Val Ser Trp Ser Gln Val Gly Val Glu Pro Ser Ile Met Gly Ile Gly Pro

        900         909         918         927         936         945         954         963         972
ATT CCA GCC ATA AAG CAA GCT GTT ACA AAA GCA GGT TGG TCA CTG GAA GAT GTT GAC ATA TTT GAA ATC AAT GAA GCC TTT
Ile Pro Ala Ile Lys Gln Ala Val Thr Lys Ala Gly Trp Ser Leu Glu Asp Val Asp Ile Phe Glu Ile Asn Glu Ala Phe

        981         990         999        1008        1017        1026        1035        1044        1053
GCA GCT GTC TCT GCT GCA ATA GTT AAA GAA CTT GGA TTA AAC CCA GAG AAG GTC AAT ATT GAA GGA GGG GCT ATA GCC TTG
Ala Ala Val Ser Ala Ala Ile Val Lys Glu Leu Gly Leu Asn Pro Glu Lys Val Asn Ile Glu Gly Gly Ala Ile Ala Leu

       1062        1071        1080        1089        1098        1107        1116        1125        1134
GGC CAC CCT CTT GGA GCA TCT GGC TGT CGA ATT CTT GTG ACC CTG TTA CAC ACA CTG GAG AGA ATG GGC AGA AGT CGT GGT
Gly His Pro Leu Gly Ala Ser Gly Cys Arg Ile Leu Val Thr Leu Leu His Thr Leu Glu Arg Met Gly Arg Ser Arg Gly

       1143        1152        1161        1170        1179        1188   1194  1200        1210        1220
GTT GCA GCC CTG TGC ATT GGG GGT GGG ATG GGA ATA GCA ATG TGT GTT CAG AGA GAA TGA CAATGT GTGTTCAGAG AGAATGAAT
Val Ala Ala Leu Cys Ile Gly Gly Gly Met Gly Ile Ala Met Cys Val Gln Arg Glu ***

      1230        1240        1250        1260        1270        1280        1290        1290        1300
TGCTTAAACT TTGAACAAGC TCAATTTCTT TTTAAACTAA TAAAGTACTA GGTTGCAATA TGTGAAATCA GAGGACCAAA GTACAGATGG
      1310        1320        1330        1340        1350        1360        1370        1380        1390
AAACCATTTC CTACATCACA AAAACCCAAG TTTACAGCTT GTACTTTACT TTAATGTGTA ATACTCAACT CACGGTACAA GACAATTGCA
      1400        1410        1420        1430
TTTAACATTG TTATAAATAA AAGGAACATC AGATCAATCA TTAAAAAAAAAAAA
```

Figure 1. *Nucleotide sequence of human CT cDNA with the deduced amino acid sequence.* Nucleotide sequence of HCT 10 is shown with the deduced amino acid sequence. The first residue of the initiator ATG triplet is designated as nucleotide number 1. The termination codon is indicated by ***. Boxed sequences, AATAAA, are the putative polyadenylation signals. Underlined amino acid sequences are identical to those obtained from sequencing of purified human CT.

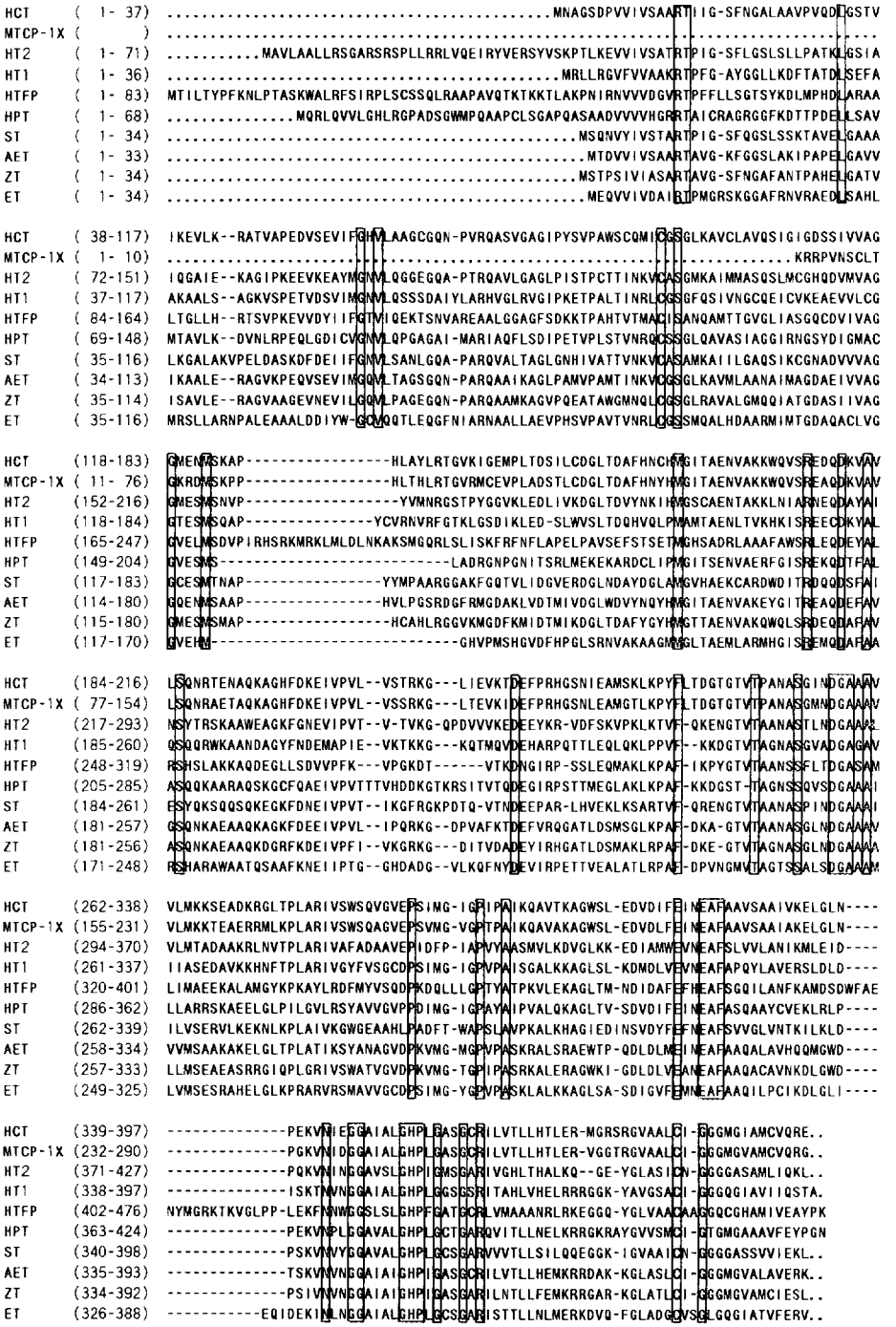

Figure 2. *Alignment of the deduced amino acid sequence of human CT with those of other thiolases.* Abbreviations used are as follows: HCT, human cytosolic thiolase; MTCP-1X, mouse t complex polypeptide-1X; HT2, human mitochondrial acetoacetyl-CoA thiolase; HT1, human mitochondrial 3-ketoacyl-CoA thiolase; HTFP, human trifunctional protein; HPT, human peroxisomal 3-ketoacyl-CoA thiolase; ST, acetoacetyl-CoA thiolase of *Saccharomyces uvarum*; AET, acetoacetyl-CoA thiolase of *Alcaligenes eutrophus*; ZT, acetoacetyl-CoA thiolase of *Zoogloea ramigera*; ET, acetoacetyl-CoA thiolase of *Escherichia coli.* Amino acid residues conserved among all the thiolases are boxed.

Table I. Thiolase activities in cells transfected with T2 and CT cDNAs

	Acetoacetyl-CoA			3-ketooctanoyl-CoA
Plasmids	-K+	+K+	+K+/-K+	
pCAGGS (-)	22.4±1.4	23.4±3.3	1.0	28
pCAGGS T2	36.5±9.3	246±86	6.7	35
pCAGGS CT	1410±90	1400±213	1.0	26

CT or T2 cDNA (4 μg) was transfected to SV40-transformed T2 null fibroblasts (GK03) with Lipofection reagent. The cells were collected and assayed 72 h after transfection. pCAGGS (-) indicates a mock cDNA transfection. Activity is represented as nmol substrate change/min/mg protein. -K+ and +K+ indicate acetoacetyl-CoA activity in the presence and absence of potassium ion, respectively.

Thiolases form an acyl-S-enzyme intermediate during their catalytic reaction. Gehring *et al.* found that pig T2 formed the intermediate at a cysteine residue (28), which corresponds to Cys126 in the human T2 sequence. The cysteine was conserved amnog the thiolases and Cys92 in CT occupies the position. Masamune *et al.* pointed out that Cys378 in ZT is also the acitive site involved in deprotonation in the reaction toward acetoacetyl-CoA formation (29,30). Indeed the positon was also occupied by the cysteine residue, in all the thiolases (Cys383 in human CT). Molecular analysis of T2 deficiency showed that alternation of amino acid residues in the highly conserved carboxy-terminal region, 346-361 in CT, of which the sequence could be summarized G-G-A-I/V-S/V-L/I-G-H-P-I/L-G-X-S/T-G-X-R resulted in instability of the thiolase protein (11, 15). The precise role of this region is unclear.

Homology search revealed that the mouse t-complex polypeptide-1 like sequence (Tcp-1x) has a 78 % homology to human CT (Fig.2). This means that CT is the same polypeptide as Tcp-1x or a protein closely related to Tcp-1x. Tcp-1x cDNA was cloned by plaque hybridization with mouse Tcp-1 cDNA but it shared only a 140 bp homology (31). Tcp-1 is considered to be a component of chaperonin for tublin and actin in the cytosol (32). Ashwarth (33) reported that Tcp-1x sequence might be that of mouse CT, as deduced from findings of the high homology with the thiolase family; the true Tcp-1-like sequence was coded in the opposite strand of the Tcp-1x cDNA. He also found that the opposite strand of human Tcp-1 gene (34) encoded a homologue sequence to the suspected CT sequence in the mouse. This thesis is correct since the nucleotide sequence of the opposite strand of most of the 3' region in human Tcp-1 gene sequence (34) matched perfectly with the 3' portion (330 bp) of the sense strand sequence of our cloned CT cDNA. These genes overlap in the 3' portion, at opposite directions. It seems likely that the human CT gene locates on chromosome 6q23-qter, because human Tcp-1 was mapped to that region (34). It must be emphasized that the suspected mouse CT cDNA apparently lacks the 5' portion of the coding sequence and there is no support for the notion that it encodes mouse CT. Our cDNA was cloned with the anti-[human CT]antibody and was confirmed to be the cDNA for cytosolic

thiolase, based on identification of amino acid sequences from purified enzyme in the deduced sequence, and by in vivo expression analysis.

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