Peroxisome proliferator-induced acyl-CoA thioesterase from rat liver cytosol: molecular cloning and functional expression in Chinese hamster ovary cells

Susanna T. ENGBERG*, Toshifumi AOYAMA†, Stefan E. H. ALEXSON‡, Takashi HASHIMOTO† and L. Thomas SVENSSON†§

*Stockholm University College of Health Sciences, Department of Biomedicine, Natural Science and Laboratory Science, Box 12773, S-112 96 Stockholm, Sweden, †Department of Biochemistry, Shinshu University, School of Medicine, Matsumoto, Nagano 390, Japan, and ‡Department of Medical Laboratory Sciences and Technology, Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge, Sweden

We have isolated and cloned a cDNA that codes for one of the peroxisome proliferator-induced acyl-CoA thioesterases of rat liver. The deduced amino acid sequence corresponds to the major induced isoform in cytosol. Analysis and comparison of the deduced amino acid sequence with the established consensus sequences suggested that this enzyme represents a novel kind of esterase with an incomplete lipase serine active site motif. Analyses of mRNA and its expression indicated that the enzyme is significantly expressed in liver only after peroxisome proliferator treatment, but isoenzymes are constitutively expressed at high levels in testis and brain. The reported cDNA sequence is highly homologous to the recently cloned brain acyl-CoA thioesterase [Broustas, Larkins, Uhler and Hajra (1996) J. Biol. Chem. **271**, 10470–10476], but subtle differences throughout the sequence, and distinct differences close to the resulting C-termini, suggest that they are different enzymes, regulated in different manners. A full-length cDNA clone was expressed in Chinese hamster ovary cells and the expressed enzyme was characterized. The palmitoyl-CoA hydrolysing activity (V_{max}) was induced approx. 9-fold to 1 μmol/min per mg of cell protein, which was estimated to correspond to a specific activity of 250 μmol/min per mg of cDNA-expressed enzyme. Both the specific activity and the acyl-CoA chain length specificity were very similar to those of the purified rat liver enzyme.

INTRODUCTION

Long-chain acyl-CoA thioesterases (EC 3.1.2.2) cleave fatty acyl-CoAs to the corresponding free fatty acids and CoA. The enzyme activity is found in all organisms and is present in several cellular compartments. Several of these activities have been purified from rat liver and brain [1–6]. In liver from rats fed with a standard chow, this enzyme activity is mainly localized to the endoplasmatic reticulum [7,8] but is induced mainly in cytosol and mitochondria by peroxisome proliferators [8], the strongest induction being in cytosol. Three different isoforms have been purified and characterized from peroxisome proliferator-treated animals. Two were localized to cytosol [1,3] and one isoform was purified from the mitochondrial compartment of induced liver [5]. One of the cytosolic enzymes has a native molecular mass of 110 kDa and was tentatively named CTE-II to distinguish it from the peroxisome proliferator-induced 47 kDa cytosolic isoform CTE-I [8]. A differential regulation of these two isoactivities by hormones and peroxisome proliferators has been established [8–10]. The induction of CTE-II, but not CTE-I, was strongly decreased in female rat liver and in castrated or adrenalectomized male rats after treatment with peroxisome proliferators [9,10].

Although the total acyl-CoA thioesterase activity of CTE-II is high, the specific content of CTE-II protein is low in cytosol of peroxisome proliferator-treated rats; previous studies showed that enzyme protein had to be enriched to be detected by Western blot analysis [3].

A constitutively expressed enzyme from rat brain, which was immunologically indistinguishable from CTE-II, has been purified and characterized [4,6]. Despite the similarity, there were several differences suggesting that they were different enzymes. For example, the brain enzyme was more heat-labile and its catalytic function was not inhibited by ATP [6]. It could not be determined, however, whether the brain and the liver enzymes were the products of the same gene. For the further characterization of CTE-II we have here isolated the corresponding cDNA by an immunological screening of a peroxisome proliferator-induced rat liver cDNA library. The deduced amino acid sequence of the cloned liver CTE-II was 96 % identical with that of the recently cloned brain enzyme [11]. The sequence of the brain enzyme, however, extended 20 amino acid residues further at the C-terminus, suggesting that the brain and liver enzymes are different, although highly homologous.

This paper describes the cDNA and its deduced amino acid sequence, corresponding to the peroxisome proliferator-induced CTE-II, and the characterization of the cDNA-expressed enzyme in Chinese hamster ovary (CHO) cells.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats (approx. 200 g) were fed with a standard pellet diet supplemented with 2 % (w/w) di-(2-ethylhexyl)phthalate (DEHP) for 10–14 days.

Abbreviations used: ACT, brain acyl-CoA thioesterase; CHO, Chinese hamster ovary; CTE-I, 47 kDa cytosolic long-chain acyl-CoA thioesterase; CTE-II, 110 kDa cytosolic long-chain acyl-CoA thioesterase; DEHP, di-(2-ethylhexyl)phthalate; EST, expressed sequence tag; MTE-I, 45 kDa mitochondrial very-long-chain acyl-CoA thioesterase.

§ To whom correspondence should be addressed. Present address: Department of Medical Biochemistry and Biophysics, Umeå University, S-901 87 Umeå, Sweden.

The nucleotide sequence data reported will appear in the EMBL Nucleotide Sequence Database under the accession number Y09332.

Materials

Acrylamide and bisacrylamide were obtained from Nacalai Tesque (Kyoto, Japan). DEHP was obtained from Kanto Chemical (Tokyo, Japan). DEAE-Toyopearl was from Tosoh Co. (Tokyo, Japan). Phenyl-Sepharose was purchased from Pharmacia (Uppsala, Sweden). Restriction enzymes were obtained from Takara (Kyoto, Japan). All acyl-CoA esters were synthesized by the mixed anhydride method as described previously [12]. All other reagents were of analytical grade and purchased from Wako Pure Chemicals (Tokyo, Japan) or Sigma (St. Louis, MO, U.S.A.).

cDNA cloning methods

Plaques (4×10^6) from a λgt11 cDNA library of clofibrate-induced male rat liver [13] were screened with an antibody against CTE-II as a probe. Anti-CTE-II antibodies were prepared as described previously [1]. Twenty positive clones were isolated and analysed. The phage DNA of these clones was purified as previously described [14]. Inserts were digested from the phage vector with *Eco*RI and subcloned into pBluescript II SK$^+$ (Stratagene, La Jolla, CA, U.S.A.). All clones were sequenced approx. 350 bases from each end.

The isolated phages from the 20 clones were used to infect *Escherichia coli* (Y1090), and the bacteria were cultured for 2 h at 37 °C. Expression of the β-galactosidase fusion protein was induced by addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM. The bacterial pellets were analysed by Western blot and the eight clones that showed immunoreactive bands on Western blot analysis were analysed more thoroughly.

DNA sequencing

After sequencing each of the eight clones 350–400 bp from both ends, an almost completely assembled sequence from overlapping cDNA fragments was obtained. On the basis of the restriction cleavage map of the assembled sequence, the complete sequencing of the longest cDNA was performed by serial deletions of the cDNA by digestion with appropriate restriction enzymes followed by subcloning into pBluescript II SK$^+$. The DNA sequencing was then performed on the DNA inserts in the plasmid by the dideoxy sequencing method [15] with the Dye Terminator cycle sequencing kit (Perkin Elmer, Hilden, Germany). The samples were subjected to electrophoresis and the gel was analysed with an Applied Biosystems 370A DNA Sequencer.

Sequence analysis was performed with GENETYX-MAC software (Software Development Co., Tokyo, Japan) and the LaserGene software package (DNAStar, London, U.K.) on Macintosh personal computers.

Northern blot analysis

Total RNA was prepared from various rat tissues from control and DEHP-treated animals by the guanidine isothiocyanate method [16]. RNA (5 μg) from each tissue sample was denatured in formaldehyde/formamide and subjected to electrophoresis in 1 % (w/v) agarose gels containing formaldehyde. The RNA was transferred to a nylon membrane (Hybond-N+, Amersham) by capillary blotting. Prehybridization and hybridization with a specific cDNA probe were performed in accordance with standard procedures. The cDNA probe was prepared by digestion of the full-length clone by *Pst*I and ^{32}P-labelled (Megalabel kit®; Takara). The hybridized bands were detected in a BAS-1500 system (Fuji Film Co., Minamiashigara, Japan).

Expression of CTE-II cDNA in CHO cells

Cells were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum, 0.1 mM non-essential amino acids (Gibco BRL, Gaithersburg, MD, U.S.A.) and 1 × antibiotic–antimycotic solution (Gibco BRL). The full-length cDNA was subcloned into the *Eco*R1 cloning site of the pCXN2 mammalian expression vector [17]. The plasmid (10 μg) was transfected into CHO cells by the calcium phosphate co-precipitation method as described previously [18]. To obtain stable transfectants, the cells were cultured with the addition of 0.5 mg/ml Geneticin disulphate (Wako Pure Chemicals). After being harvested, the cells were washed with 0.5 ml of PBS and then sonicated. After centrifugation, the supernatant was analysed by Western blotting and assayed for enzyme activity.

Immunoprecipitation

CHO cell lysate was prepared as described above. A 50 μl aliquot of the lysate was incubated with 5 μl of the antibody against CTE-II for 1 h at 25 °C. After centrifugation and pelleting of the immunoprecipitate, the enzyme activity was measured in the supernatant as described previously [1].

Lipid analysis

Total lipids were extracted with chloroform/methanol (2:1, v/v) from CHO cells grown to 60–70 % confluence on 8 cm² dishes, after incubation with 1.4 nmol of [^{14}C]palmitate for 1 or 2.5 h, in a volume of 1 ml. The neutral lipids were separated on TLC plates with hexane/diethyl ether/acetic acid (80:20:1, by vol.). The radioactive spots corresponding to neutral lipids were detected and quantified with a BAS-1500 system. The plates were then transferred to a solvent system composed of chloroform/methanol/water (65:25:4, by vol.) in which the polar compounds were separated in the same dimension. After separation the polar lipids were detected and quantified with the BAS-1500 system.

Phospholipids were analysed from control and transfected cells cultured without the addition of [^{14}C]palmitate. The phospholipids were separated in a two-dimensional system [19], and after detection of the spots they were scraped off and the phosphorus content was determined as previously described [20].

Purification of CTE-II

Livers from 10 rats (approx. 140 g) that had been fed with DEHP for 10 days were cut into pieces and homogenized in a Potter–Elvehjem glass–Teflon homogenizer in 0.25 M sucrose containing 1 mM EDTA. The homogenate was centrifuged for 5 min at 5000 ***g***. The supernatant was centrifuged for 1 h at 100000 ***g*** to obtain the supernatant (cytosolic fraction). CTE-II was purified by a protocol modified from Miyazawa et al. [1] and Yamada et al. [3].

The cytosolic fraction was heat treated at 61–62 °C for 3 min. After the fraction had been cooled on ice to 10 °C, the pH was adjusted to 5.4 by the addition of 1 M acetic acid. After centrifugation at 10000 ***g*** for 10 min the supernatant was adjusted to pH 7.8 by the addition of 1 M KOH. This fraction was treated with $(NH_4)_2SO_4$ and the protein precipitating between 35–65 % saturation with $(NH_4)_2SO_4$ was collected. The protein was dissolved in 20 mM potassium phosphate, pH 7.8, containing 1 mM EDTA (buffer A) and dialysed against 10 mM potassium phosphate, pH 7.8, containing 1 mM EDTA. The dialysed fraction was applied to a TSK gel DEAE toyopearl 650 M (Tosoh) column (90 mm × 55 mm) equilibrated with buffer A. The enzyme was eluted with a 400 ml linear gradient, ranging from 0 to 0.5 M

NaCl in buffer A. Fractions with enzymic activity were collected and applied to a phenyl-Sepharose CL 4B (Pharmacia) column (51 mm × 28 mm), equilibrated with buffer A containing 200 mM NaCl. The enzyme was eluted by a 200 ml linear gradient of 0–60 % (v/v) ethylene glycol in buffer A. The active fractions were pooled and applied to a TSK gel AF-blue Toyopearl 650 ML (Tosoh) column (92 mm × 18 mm), equilibrated with buffer A containing 20 % ethylene glycol. The column was washed with 5 column volumes of buffer A containing 20 % ethylene glycol, 10 μM NADH and 10 μM NADPH. The enzyme was eluted with 10 μM CoA and 20 μM dithiothreitol in buffer A containing 20 % ethylene glycol. Fractions with enzyme activity were collected and concentrated by centrifugation in an Ultracent-30 device (Tosoh). The pure enzyme preparation was adjusted to 50 % ethylene glycol and stored at −80 °C.

Peptide sequencing

Purified CTE-II was cut out from a gel after SDS/PAGE (10 % gel) and digested with endoproteinase Lys-C in the stacking gel of a second SDS/PAGE (15 % gel) in accordance with the method of Cleveland et al. [21]. After electrophoresis the resulting peptides were electrotransferred to a PVDF membrane (Bio-Rad). The membrane was stained with Amido Black, and the peptide sequence of the major stained band was determined with a model 477A gas-phase sequenator (Applied Biosystems) and an in-line model 120A phenylthiohydantoin analyser.

Other methods

Acyl-CoA thioesterase activity was routinely followed spectrophotometrically with 5,5′-dithiobis-(2-nitrobenzoic acid), as described previously [5]. The appropriate acyl-CoA was pre-incubated and the reaction was started by the addition of enzyme. Protein was determined with the Coomassie protein assay reagent (Pierce) with BSA as standard.

The estimation of kinetic parameters was performed by fitting the experimental data to the Michaelis–Menten equation; the unknown parameters were solved numerically by the computer program KaleidaGraph 3.0 for Apple Macintosh.

RESULTS

Screening of cDNA clones

The immuno-screening with anti-CTE-II of the λgt11 cDNA library from clofibrate-induced rat liver resulted in the isolation of 20 positive clones, after screening 4.5×10^6 plaques. The verification of positive clones isolated was performed by infecting *E. coli* with the purified phages and subsequently analysing the isopropyl β-D-thiogalactoside-induced expression product by immunoblotting. Of the 20 initial clones, eight gave a positive signal for CTE-II and were analysed further. The positive clones were sequenced 350–400 bases from both ends by using SK and KS primers (Stratagene), and 100 % sequence identity in the overlapping sequences suggested that all clones encoded the same protein. Two of the clones contained the entire coding region and the longest clone was completely sequenced on both strands (Figure 1). The clone contained a 1346 bp insert with 12 bp of 5′ untranslated region and an open reading frame of 1014 bp encoding a protein of 338 amino acid residuess with a calculated molecular mass of 37.6 kDa (Figure 2). A putative polyadenylation signal, AATAAA, was located at position 1309, 21 bp upstream from the poly(A) tail.

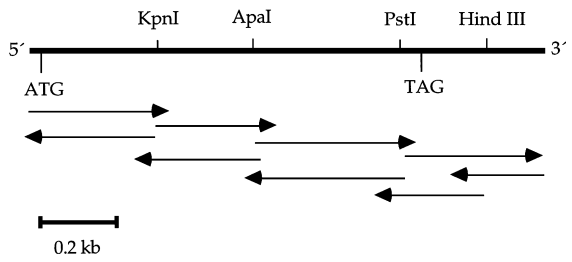

Figure 1 Restriction map and sequencing strategy

The longest CTE-II cDNA was digested by the indicated restriction enzymes; the resulting fragments were subcloned into pBluescript II SK$^+$ and sequenced, as indicated by the arrows, from the new ends by using SK, KS or T7 primers.

```
       GTCGCAGCCAAGATGTCCGGTCCCACCACCGACACGCCGGCCGCCATCCAGATCTGCCGG    60
                 M  S  G  P  T  T  D  T  P  A  A  I  Q  I  C  R
       ATCATGCGTCCGGATGATGCCAACGTGGCCGGCAATGTTCACGGAGGGACCATTCTAAAG   120
 17    I  M  R  P  D  D  A  N  V  A  G  N  V  H  G  G  T  I  L  K
       ATGATCGAGGAGGCTGGGGTCATCATCAGCACCCGGCACTGTAACAGCCAGAATGGGGAG   180
 37    M  I  E  E  A  G  V  I  I  S  T  R  H  C  N  S  Q  N  G  E
       CGCTGTGTGGCTGCCCTGGCCCGGGTGGAGCGCACTGACTTCCTGTCGCCCATGTGCATC   240
 57    R  C  V  A  A  L  A  R  V  E  R  T  D  F  L  S  P  M  C  I
       GGTGAGGTGGCTCACGTCAGCGCAGAGATCACCTATACTTCCAAGCACTCTGTGGAGGTC   300
 77    G  E  V  A  H  V  S  A  E  I  T  Y  T  S  K  H  S  V  E  V
       CAGGTCCACGTGTTGTCGGAGAACATCCTCACAGGTACCAAAAAGCTGACCAATAAGGCC   360
 97    Q  V  H  V  L  S  E  N  I  L  T  G  T  K  K  L  T  N  K  A
       ACCTTGTGGTATGTGCCCCTGTCATTGAAGAATGTGGACAAGGTCCTTGAGGTGCCTCCT   420
117    T  L  W  Y  V  P  L  S  L  K  N  V  D  K  V  L  E  V  P  P
       ATTGTGTATTTACGGCAGGAACAGGAGGAGGAGGGTCGGAAACGCTATGAAGCCCAGAAG   480
137    I  V  Y  L  R  Q  E  Q  E  E  E  G  R  K  R  Y  E  A  Q  K
       CTAGAACGCATGGAGACCAAGTGGAGGAACGGAGACATTGTCCAGCCCATCCTGAACCCA   540
157    L  E  R  M  E  T  K  W  R  N  G  D  I  V  Q  P  I  L  N  P
       GAGCCGAACACAGTGAGCTACAGCCAGTCCAGCCTGATCCACCTGGTGGGGCCCTCAGAC   600
177    E  P  N  T  V  S  Y  S  Q  S  S  L  I  H  L  V  G  P  S  D
       TGCACTCTTCATGGCTTCGTGCACGGAGGTGTCACCATGAAGCTCATGGATGAGGTGGCC   660
197    C  T  L  H  G  F  V  H  G  G  V  T  M  K  L  M  D  E  V  A
       GGGATTGTGGCTGCGCGCCACTGCAAGACCAATATAGTGACTGCCTCTGTGGATGCTATT   720
217    G  I  V  A  A  R  H  C  K  T  N  I  V  T  A  S  V  D  A  I
       AATTTCCATGACAAGATCCGGAAAGGCTGTGTCATCACCATCTCTGGACGCATGACCTTC   780
237    N  F  H  D  K  I  R  K  G  C  V  I  T  I  S  G  R  M  T  F
       ACAAGCAATAAGTCTATGGAAATTGAGGTCCTGGTGGACGCTGACCCTGTGGTGGACAAC   840
257    T  S  N  K  S  M  E  I  E  V  L  V  D  A  D  P  V  V  D  N
       TCACAGAAGCGCTACCGGGCTGCCAGTGCCTTCTTCACCTACGTGTCCCTGAATCAGGAG   900
277    S  Q  K  R  Y  R  A  A  S  A  F  F  T  Y  V  S  L  N  Q  E
       GGCAAGCCGCTGCCTGTGCCTCAGCTTGTGCCGGAGACGGAGGACGAGAAGAAGCGTTTT   960
297    G  K  P  L  P  V  P  Q  L  V  P  E  T  E  D  E  K  K  R  F
       GAAGAAGGCAAAGGCCGCTATCTGCAGATGAAGGCGAAGCGACAGGGCCATACAGAGCCT  1020
317    E  E  G  K  G  R  Y  L  Q  M  K  A  K  R  Q  G  H  T  E  P
       CAGCCCTAGATGTCTTCCTCCCTCCCATCCTGTCCCGTCCTGGGTCAGCACAGTTGTGGC  1080
337    Q  P  *
       AGTAGTCCCGTGTGCAGTCACTTAGAAGTCGCCCCCTTGGCCAAACCCCGATTTCCTTTG  1140
       AGAGCTGGTGTTGTGAAGTACCGTGTGGCAGTGTTACCTGTGGCCTGTTCCCAAAACCTG  1200
       TGCACCAAAGCTTTATTTATATCCCTCCAGTCCCTGCTCCCATGTTGTCCCAAAGGCCAT  1260
       CGTGGACACCAGAGCACACTGACTGGCCTGGAGAAGCCAGCACCACTAATAAAGCTGCTG  1320
       TCTGGCTGGAAAAAAAAAAAAAAAA                                     1346
```

Figure 2 Nucleotide sequence and deduced amino acid sequence of CTE-II

The doubly underlined amino acid sequence corresponds to the proteolytically derived and sequenced peptide from purified CTE-II. The polyadenylation site at nucleotide positions 1308–1313 and the poly(A) tail are underlined. The stop codon is identified by an asterisk.

Comparison of the amino acid sequence with that of purified CTE-II

To verify that the isolated clone coded CTE-II, we determined the amino acid sequence of a peptide isolated after digestion of purified CTE-II with endoproteinase Lys-C. The enzyme was purified by the methods of Miyazawa et al. [1] and Yamada et al. [3] (results not shown). Both methods resulted in the purification of the same protein; we decided to purify the enzyme by a modified protocol, as described in the Experimental section. SDS/PAGE and Western blotting analysis showed that the purified enzyme had an apparent molecular mass of 40 kDa. Analysis of the proteolytic fragment resulted in the 16-amino-acid sequence SMEIEVLVDADPVVDN, which corresponds to the deduced amino acid sequence between residues 261 and 276 in the CTE-II clone (Figure 2, doubly underlined).

Position	1	2	3	4	5	6	7	8	9	10
Consensus	[LIV]	X	[LIVFY]	[LIVST]	G	[HYWV]	S	X	G	[GSTAC]
CTE-II	I-189	H	L	V	G	P	S	D	C	T-198

Figure 3 Comparison of amino acid residues 189–198 in the CTE-II sequence with the suggested lipase serine active site sequence motif

The suggested alternatives in the consensus sequence from the Prosite sequence motif database are presented and aligned with the putative active site of CTE-II. Amino acid residues that do not fit into the consensus sequence are underlined.

A

```
                                                          ∇2      1036
CTE-II   TATCTGCAGATGAAGGCGAAGCGACAGGGCCATACAGAGCCTCAGCCCTAGATGTCTT
ACT      TATCTGCAGATGAAGGCGA-CAGACAGGGCCACACAGAGCCTCAGCCCTAGGTGTCTT
                           Δ1                                1115

                                                            1096
CTE-II   CCTCCCTCCCATCCTGTCCCGTCCTGGGTCAGCACAGTTGTGGCAGTAGTCCCGTGTGCA
ACT      CC----------TCCTGTCCC-----GGGTCAGCACAGTTGTGGCAATAG-CCAGTATGCA
                                                            1159

                        1116
CTE-II   GTCACTTAGAAGTCGCCCCC
ACT      GTCACTTAGAAATTGCCCCC
            Δ3          1179
```

B

```
                    338
CTE-II   YLQMKAKRQGHTEPQP*
ACT      YLQMKATDRATQSLSPRCLPPVPGQHSCGNSQYAVT*
                                          358
```

Figure 4 Comparison of the CTE-II sequence and the rat brain ACT sequence

(**A**) The region of the CTE-II nucleotide sequence that contains the highest divergence from the ACT sequence is presented. Between nucleotides 1077 and 1080 (arrowhead 1) in the brain ACT sequence there exists an apparent deletion of one nucleotide, which causes the brain sequence to continue beyond the stop codon in the liver CTE-II sequence (arrowhead 2). Between the stop codon for CTE-II (arrowhead 2) and brain ACT (arrowhead 3) there are several distinct differences in the nucleotide sequence. Five of eight CTE-II cDNA clones covered the presented region; they were all 100% identical in this region. (**B**) The nucleotide sequences in (**A**) have been translated into their amino acid sequence and compared. The respective stop codons are identified by asterisks.

Homology search

Analysis of the cloned CTE-II sequence showed the absence of a typical serine esterase active site motif, GXSXG [22]. Instead, an incomplete sequence motif with 75% identity to the lipase serine active site consensus sequence (Prosite sequence motif database) was found between residues 189 and 198 (Figure 3). The second obligatory Gly residue was replaced with Cys, and the residue at position 6 was replaced with the non-polar Pro in the CTE-II sequence. Homology searches with the coding region of the CTE-II clone in DNA databases showed significant similarity to an acyl-CoA thioesterase (ACT) recently cloned from rat brain [11] (Figures 4A and 4B). The 338-residue sequence of CTE-II was 95.9% identical with the corresponding sequence of brain ACT, and the first 328-residue sequence was 98.5% identical. The absence of an A at ACT residue 1077 (Figure 4A, arrowhead 1) causes a shift in the reading frame of the brain ACT, which elongates the protein to 358 residues (Figure 4B). Five of the eight positive CTE-II clones covered this region, and sequencing revealed that they were 100% identical with the full-length CTE-II clones in this region (results not shown). Before the deduced amino acid residue 329 there are 47 nucleotides that differ between the two sequences. Of these, 42 are 'silent' substitutions in the third position of the codons. Searches in

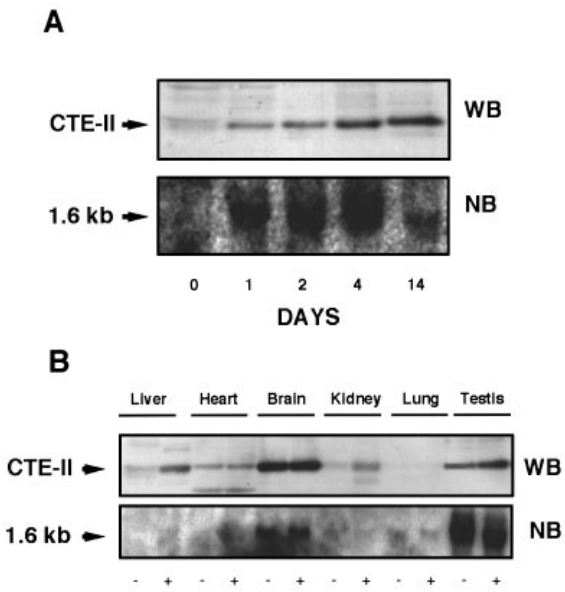

Figure 5 Analysis of the effect of treatment with DEHP on the expression of CTE-II in liver and the tissue specificity of CTE-II

(**A**) Liver homogenates (10 μg) and total liver RNA (5 μg) from rats treated with DEHP for the indicated periods were analysed by Western blotting and Northern blotting as described in the Experimental section: upper panel, Western blot analysis (WB); lower panel, the corresponding Northern blot analysis (NB). Each blot is representative of three independent experiments. (**B**) The indicated tissue homogenate (10 μg) and total RNA (5 μg) from control rats (−) and rats treated with DEHP for 14 days (+) were analysed by Western blotting (upper panel) and Northern blotting (lower panel). The Western blot is representative of two independent blots and the Northern blot is representative of three independent blots.

sequence databases revealed that two different 'expressed sequence tags' (ESTs) from mouse brain are 100% identical with the CTE-II sequence (EST accession no. W54982) and the brain ACT sequence (EST accession no. W14302) respectively. This region of the CTE-II and ACT cDNA sequences contained 17 conservative base exchanges, suggesting that both isoenzymes are expressed in mouse brain, but none is expressed in mouse liver even after clofibrate treatment [23]. On the basis of our data we propose that the rat liver and brain isoenzymes are products of different genes, which show different regulations of expression.

Additionally, we found a strikingly high sequence identity of CTE-II to several human ESTs cloned from different tissues such as brain, placenta, monocytes and senescent fibroblasts, possessing more than 90% sequence identity (for example, accession numbers W44631, T74295, M62036, T47596, F12498). The extremely high sequence conservation between CTE-II, brain ACT and the human and mice ESTs suggests that the genes coding for CTE-II and brain ACT respectively are due to evolutionarily recent gene duplications and divergence.

Tissue specificity and effect of DEHP treatment

Expression of CTE-II protein and mRNA was first analysed in liver at different time points after the addition of DEHP to the diet (Figure 5A). No signal was seen in livers from untreated rats, but the expression of CTE-II protein was found only 1 day after the addition of DEHP to the food and reached a maximum at 14 days (Figure 5A, upper panel). The expression of specific mRNA in liver was found to correspond to the increased expression of CTE-II protein until 4 days of the treatment, but the amount of mRNA decreased significantly after 14 days of treatment

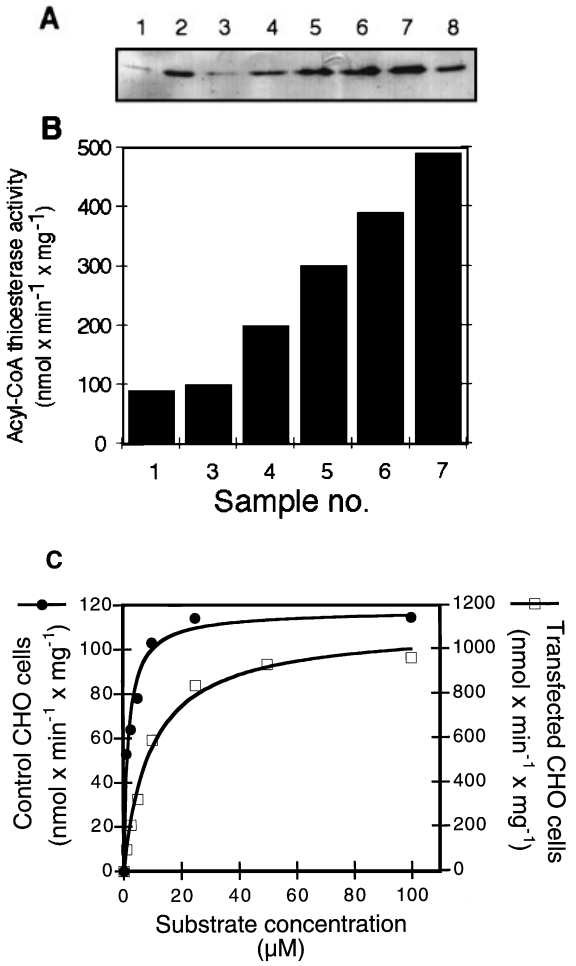

Figure 6 Expression of recombinant CTE-II in CHO cells

(**A**) Control CHO cells and CHO cells transfected with CTE-II cDNA, as described in the Experimental section, were analysed by Western blotting. A 5 μg sample of each cell extract was applied to each well. Lane 1, control CHO cells; lanes 2 and 8, 10 ng of purified CTE-II; lanes 3–7, transfected CHO cells with various degrees of expression of recombinant CTE-II. (**B**) Acyl-CoA thioesterase activities in CHO cell extracts, corresponding to lanes 1 and 3–7 in (**A**), were analysed with 10 μM palmitoyl-CoA. (**C**) The dependence of palmitoyl-CoA thioesterase activity on substrate concentration was analysed between 1 and 100 μM palmitoyl-CoA in control CHO cells (●) and in CHO cells transfected with CTE-II (□, same cell line as in Table 1.)

(Figure 5A, lower panel). In brain and testis, high levels of CTE-II expression were found in both the untreated and the DEHP-treated conditions (Figure 5B). The mRNA level in brain and testis was much higher than in liver after DEHP treatment for 14 days, and it was not possible to quantify them within a linear range. However, we analysed the samples from liver treated with DEHP for 4 days and compared them with samples from brain and testis treated with DEHP for 14 days; we found that the signals were 6-fold and 25-fold higher in brain and testis respectively than in liver. The lack of correlation between the amount of enzyme protein and specific mRNA in DEHP-treated liver, brain and testis might be explained by different degradation rates of enzyme protein in the different tissues. The relatively slow induction of CTE-II protein in liver suggests that the turn-over rate might be slow, resulting in the accumulation of CTE-II protein.

Table 1 Comparison of acyl-CoA chain-length specificities of CTE-II in control and transfected CHO cells

Control CHO cells and transfected cells expressing CTE-II were analysed for acyl-CoA chain length specificity at a substrate concentration of 10 μM. Each value represents the mean activity ± S.E.M. for three different culture dishes.

	CTE-II activity (nmol/min per mg)	
Substrate	Control cells	Transfected cells
Octanoyl-CoA	79.3 ± 3	257.3 ± 8
Decanoyl-CoA	79.7 ± 2	418.7 ± 36
Lauroyl-CoA	80.3 ± 3	429.6 ± 10
Myristoyl-CoA	86.1 ± 3	494.6 ± 8
Palmitoyl-CoA	78.9 ± 5	437.5 ± 19
Stearoyl-CoA	50.0 ± 3	319.7 ± 20

cDNA expression in CHO cells

To verify that the cDNA encoded the functional protein, cDNA expression was performed in CHO cells. We constructed stable transfectants, as described in the experimental section. The endogenous acyl-CoA thioesterase activity was high in CHO cells, approx. 100 nmol/min per mg towards palmitoyl-CoA, and a Western blot analysis of control cells showed that a hamster homologue of CTE-II was expressed endogenously in these cells. When immunoprecipitation was performed with the antibody against CTE-II, almost all activity was precipitated in both control and transfected cells, with the residual activity being less than 10 nmol/min per mg. Western blot analysis showed identical mobilities of the purified and the expressed CTE-II in different transfected cell lines, i.e. 40 kDa (Figure 6A). Figure 6(B) shows that the acyl-CoA thioesterase activity is correlated with the degree of expression of CTE-II in Figure 6(A). The substrate specificity of the cDNA-expressed CTE-II (Table 1) was similar to that of the purified enzyme [1]; no inhibition at high substrate concentration was seen (Figure 6C). The V_{max} measured with palmitoyl-CoA as substrate was 9-fold higher in transfected cells than in control cells (Figure 6C). The apparent K_m and V_{max} values for the enzyme expressed in CHO cell were 10 μM and 1090 nmol/min per mg of cell protein respectively. In control cells, the corresponding values were 2 μM and 120 nmol/min per mg of cell protein. In a Western blot analysis we estimated the concentration of cDNA-expressed CTE-II to be 20 ng per 5 μg of cell protein (Figure 6A, lane 7), resulting in an estimated V_{max} for the cDNA-expressed CTE-II of 250 μmol/min per mg, which is similar to previously reported values for CTE-II [1,3].

To investigate whether CTE-II is involved in a specific acyl-CoA-dependent acyltransferase reaction as an acyl-group donor, we analysed the overall phospholipid contents and compositions in control and transfected cells. We also analysed the effect of expression of CTE-II in CHO cells on the specific incorporation of [^{14}C]palmitate in various lipid fractions in the cells and in the culture medium. The radioactive palmitate was rapidly incorporated into cell lipids and to some extent into lipids in the culture medium, but neither experiment showed significant differences between control and transfected cells (results not shown).

DISCUSSION

A cDNA corresponding to CTE-II was isolated and characterized. We concluded that the cDNA coded the correct enzyme on the basis of the internal peptide sequence of CTE-II and a

comparison with the deduced amino acid sequence. Expression of the cDNA in CHO cells greatly increased the acyl-CoA thioesterase activity in these cells. The expressed activity could be precipitated with anti-CTE-II antibodies, which recognized a band of identical size to that of CTE-II as determined by Western blot analysis. CTE-II is apparently a different gene product from the recently cloned brain ACT, because of the pronounced differences in the C-terminal region. In addition, the fact that 42 out of 47 nucleotide differences before residue 329, evenly distributed throughout the sequence, were in the third position of the respective amino-acid-coding triplets, not resulting in amino acid substitutions, indicated that the two enzymes are different. The identical nucleotide sequences of two mouse ESTs, corresponding to the N-terminal parts of CTE-II and brain ACT, also strongly suggest that the two isoforms are different gene products. The sequence conservation between CTE-II, brain ACT and the human and mouse ESTs indicates that these enzymes possess important but possibly different physiological functions. To provide different biological regulation in liver and brain respectively, evolution resulted in gene duplication; the functional characterization of these gene products and isolation of the respective genes will be of great importance in understanding their physiological roles.

We have previously shown that two different acyl-CoA thioesterase isoforms are expressed in liver cytosol after treatment of rats with peroxisome proliferators, i.e. CTE-I and CTE-II [8]. These are structurally different, because antibodies against CTE-II do not recognize CTE-I, and vice versa [3]. However, antibodies against the 45 kDa mitochondrial very-long-chain acyl-CoA thioesterase MTE-I recognize the slightly larger CTE-I (47 kDa), and we previously partly purified a possible mitochondrial counterpart to CTE-II with the same native molecular mass [5]. The antibody against CTE-II precipitated approx. 50% of the acyl-CoA thioesterase activity in rat liver mitochondria after treatment with DEHP [1] and showed an immunoreactive band of identical size to CTE-II on Western blot analysis of partly purified MTE-II, the 110 kDa mitochondrial long-chain acyl-CoA thioesterase (results not shown). However, so far we have not been able to isolate cDNAs that coded for a distinct mitochondrial isoform. The reason is possibly the limited number of immunopositive clones that we were able to isolate. We are currently attempting to complete the isolation of the mitochondrial isoenzyme to establish the functional and structural relationship of this apparently dual localization.

In contrast with thioesterase I and II [24–26], which terminate fatty acid synthesis *in vivo* but hydrolyse acyl-CoA *in vitro*, several other acyl-CoA-hydrolysing enzymes have recently been characterized as insensitive to classical serine esterase/lipase inhibitors (e.g. PMSF) but sensitive to the thiol-reacting agent *p*-chloromercuribenzoate [3,5,27,28]. Molecular cloning of palmitoyl-protein thioesterase, which was not inhibited by serine esterase inhibitors, unexpectedly revealed a putative active site motif similar to the serine active site in lipases [29]. Also, CTE-II has a putative lipase serine active site motif, although less complete, and is not sensitive to PMSF but is sensitive to P-chloromercuribenzoate. In addition we have recently cloned MTE-I and CTE-I (L. T. Svensson, S. T. Engberg, T. Aoyama, S. E. H. Alexson and T. Hashimoto, unpublished work). They both possess putative lipase serine active sites, although they are not sensitive to the serine esterase inhibitors difluorophosphate or PMSF. These enzymes share no significant overall similarity (except between MTE-I and CTE-I) but their putative serine esterase motifs, the resistance to serine esterase inhibitors and the sensitivity to thiol-reactive agents suggest that they represent a novel kind of mechanism for thioester hydrolysis.

The analysis of the deduced amino acid sequence of CTE-II and comparison with the Prosite sequence motif database revealed an area that is a candidate region for the catalytic active site domain. This sequence contains the putative nucleophile involved in ester bond hydrolysis, i.e. Ser-195 (or Cys-197) and has the hydrophobic properties needed to bind the substrate. However, the sequence differs at one position: the second obligatory Gly residue, regarded as essential for lipases and several other carboxyl esterases, is replaced with a Cys. Site-directed mutagenesis experiments should be performed to investigate the importance of the Cys-197 and Pro-194 residues for acyl-CoA thioesterase activity.

Northern blot analysis showed that the CTE-II mRNA was rapidly induced after treatment of rats with a peroxisome proliferator, which might indicate that the regulation of CTE-II is directly dependent on a mechanism mediated by the peroxisome proliferator-activated receptor, a member of the steroid hormone receptor superfamily [30].

The physiological function of CTE-II (and brain ACT) is not clear. One possible function might be an involvement in the synthesis of membrane phospholipids, on the basis of its co-induction with phospholipid synthesis in liver after treatment with peroxisome proliferators, and its constitutive expression in brain. However, expression with an almost 10-fold increase in CTE-II activity in CHO cells, followed by an analysis of the incorporation of [^{14}C]palmitate in various lipids, partly ruled out the involvement of CTE-II in these processes. The high constitutive expression in brain and testis and in ovary cells suggests a possible involvement in steroid synthesis, an aspect that deserves further attention.

In conclusion, the results presented here, together with those of Broustas et al. [11], suggest that this peroxisome proliferator-induced long-chain acyl-CoA thioesterase expressed in liver and the brain acyl-CoA thioesterase are of great interest for future studies on functional aspects of apparent thioester hydrolysis and tissue specific expression. Isolation of the orthologous human cDNA species may help us to understand the physiological relevance of these enzymes, as defects in their genes might be manifested in a recognizable phenotype.

We thank M. Souri for invaluable help, S. Miyazawa for the peptide sequencing and Dr. T. Taketomi for phospholipid analysis. This investigation was supported by a Grant-in-aid C for Scientific Research from the Ministry of Education, Science and Culture in Japan, a research grant for intractable diseases from the Ministry of Health and Welfare of Japan and a grant from the Swedish Natural Science Research Council. L. T. S. was supported by a postdoctoral fellowship from the Japan Society for the Promotion of Science and a Grant-in-aid from the Ministry of Education, Science and Culture in Japan.

REFERENCES

1 Miyazawa, S., Furuta, S. and Hashimoto, T. (1981) Eur. J. Biochem. **117**, 425–430
2 Alexson, S. E. H., Mentlein, R., Wernstedt, C. and Hellman, U. (1993) Eur. J. Biochem. **214**, 719–727
3 Yamada, J., Matsumoto, I., Furihata, T., Sakuma, M. and Suga, T. (1994) Arch. Biochem. Biophys. **308**, 118–125
4 Broustas, C. G. and Hajra, A. K. (1995) J. Neurochem. **64**, 2345–2353
5 Svensson, L. T., Alexson, S. E. and Hiltunen, J. K. (1995) J. Biol. Chem. **270**, 12177–12183
6 Yamada, J., Furihata, T., Tamura, H., Watanabe, T. and Suga, T. (1996) Arch. Bichem. Biophys. **326**, 106–114
7 Mentlein, R., Rix-Matzen, H. and Heymann, E. (1988) Biochim. Biophys. Acta **964**, 319–328
8 Svensson, L. T., Wilcke, M. and Alexson, S. E. (1995) Eur. J. Biochem. **230**, 813–820
9 Kawashima, Y., Katoh, H. and Kozuka, H. (1982) Biochim. Biophys. Acta **712**, 48–56
10 Kawashima, Y., Katoh, H. and Kozuka, H. (1983) Biochim. Biophys. Acta **750**, 365–372

11 Broustas, C. G., Larkins, L. K., Uhler, M. D. and Hajra, A. K. (1996) J. Biol. Chem. **271**, 10470–10476
12 Wieland, T. and Rueff, L. (1953) Angew. Chem. **65**, 186–187
13 Aoyama, T., Ueno, I., Kamijo, T. and Hashimoto, T. (1994) J. Biol. Chem. **269**, 19088–19094
14 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15 Sanger, E., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
16 Davis, L. G., Dibner, M. D. and Battey, J. F. (1986) Basic Methods in Molecular Biology, Elsevier, New York
17 Niwa, H., Yamamura, K.-I. and Miyazaki, J.-I. (1991) Gene **108**, 193–200
18 Kamijo, T., Aoyama, T., Miyazaki, J. and Hashimoto, T. (1993) J. Biol. Chem. **268**, 26452–26460
19 Sugiyama, E., Uemura, K.-I., Hara, A. and Taketomi, T. (1990) Biochem. Biophys. Res. Commun. **169**, 673–679
20 Bartlett, G. R. (1959) J. Biol. Chem. **234**, 466–468
21 Cleveland, D. W., Fisher, S. G., Kirchner, M. W. and Laemmli, U.K. (1977) J. Biol. Chem. **252**, 1102–1106
22 Derewenda, Z. S. and Sharp, A. M. (1993) Trends Biochem. Sci. **18**, 20–25
23 Katoh, H., Nakajima, S., Kawashima, Y., Kozuka, H. and Uchiyama, M. (1984) Biochem. Pharmacol. **33**, 1081–1085
24 Naggert, J., Williams, B., Cashman, D. P. and Smith, S. (1987) Biochem. J. **243**, 597–601
25 Naggert, J., Witkowski, A., Mikkelsen, J. and Smith, S. (1988) J. Biol. Chem. **263**, 1146–1150
26 Smith, S. (1994) FASEB J. **8**, 1248–1259
27 Lehner, R. and Kuksis, A. (1993) J. Biol. Chem. **268**, 24726–24733
28 Camp, L. A. and Hofmann, S. L. (1993) J. Biol. Chem. **268**, 22566–22574
29 Camp, L. A., Verkruyse, L. A., Afendis, S. J., Slaughter, C. A. and Hofmann, S. L. (1994) J. Biol. Chem. **269**, 23212–23209
30 Issemann, I. and Green, S. (1990) Nature (London) **347**, 645–650

Received 10 October 1996/5 December 1996; accepted 10 December 1996