Molecular cloning and characterization of a mitochondrial peroxisome proliferator-induced acyl-CoA thioesterase from rat liver

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We have previously reported the purification and characterization of the peroxisome proliferator-induced very-long-chain acyl-CoA thioesterase (MTE-I) from rat liver mitochondria [L. T. Svensson, S. E. H. Alexson and J. K. Hiltunen (1995) J. Biol. Chem. **270**, 12177–12183]. Here we describe the cloning of the corresponding cDNA. One full-length clone was isolated that contained an open reading frame of 1359 bp encoding a polypeptide with a calculated molecular mass of 49 707 Da. The deduced amino acid sequence contains a putative mitochondrial leader peptide of 42 residues. Expression of the cDNA in Chinese hamster ovary cells, followed by immunofluorescence, immunoelectron microscopy and Western blot analyses, showed that the product was targeted to mitochondria and processed to a mature protein of 45 kDa, which is similar to the molecular mass of the protein isolated from rat liver mitochondria. The recombinant enzyme showed the same acyl-CoA chain-length specificity as the isolated rat liver enzyme. Sequence analysis showed no similarity to known esterases, but a high degree (approx. 40 %) of identity with bile acid-CoA:amino acid *N*-acyltransferase cloned from human and rat liver. A putative active-site serine motif (Gly-Xaa-Ser-Xaa-Gly) of several carboxylesterases and lipases was identified. Western and Northern blot analyses showed that MTE-I is constitutively expressed in heart and is strongly induced in liver by feeding rats with di(2-ethylhexyl)phthalate, a peroxisome proliferator, suggesting a role for the enzyme in lipid metabolism.

INTRODUCTION

Long-chain acyl-CoA hydrolase (EC 3.1.2.2) cleaves acyl-CoA to the corresponding free fatty acid and CoA. The enzyme's activity is widely distributed among organisms and cell types. In rat liver the activity is found in several cellular compartments [1–3] and the activity is highly regulated by hormonal and dietary conditions [4,5]. In normal liver the main activity is found in the endoplasmic reticulum [3,6]. This microsomal activity is due mainly to two enzymes [7,8], of which one is a member of the liver carboxylesterase multigene family [9]. The cDNA corresponding to microsomal carboxylesterase/acyl-CoA thioesterase was cloned recently [10,11] but the physiological functions remain so far unknown. Interestingly, after treatment of rats with various peroxisome proliferators, the hydrolytic activity towards long-chain acyl-CoAs was induced mainly in the cytosolic compartment [2] and in the mitochondria [3]. The activity induced in the cytosol was shown to be due to two enzymes, tentatively named CTE-I and CTE-II, which have been purified [2,12]. A cDNA and deduced amino acid sequence for CTE-II was recently established [13]. Also in mitochondria the increased activity after treatment with peroxisome proliferators was shown to be due to the induction of two acyl-CoA thioesterases [6]. A 45 kDa enzyme was purified to apparent homogeneity and found to be most active with long- and very-long-chain acyl-CoAs; it was tentatively named MTE-I [14]. Antibodies against MTE-I recognized a 46 kDa isoenzyme in cytosol (CTE-I) and a 47 kDa isoenzyme in peroxisomes (PTE-I) [6]. The molecular cloning of MTE-I and CTE-I is described in this paper. The deduced amino acid sequences revealed a high degree of similarity but contained distinct differences that explain the different subcellular localizations of the enzymes.

The cloning and characterization of these cDNA species are important steps towards revealing the metabolic role of the apparent acyl-CoA hydrolysis caused by peroxisome proliferators.

EXPERIMENTAL

Animals

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

Materials

DEHP was obtained from Kanto Chemical (Tokyo, Japan). Restriction enzymes were obtained from Takara (Kyoto, Japan). Reagents for DNA and peptide sequencing were obtained from Applied Biosystems (Tokyo, Japan). All acyl-CoA esters were synthesized by the mixed-anhydride method as described previously [15]. All other reagents were of analytical grade and

Abbreviations used: BAT, bile acid-CoA: amino acid *N*-acyltransferase; CHO, Chinese hamster ovary; CTE-I, 46 kDa cytosolic long-chain acyl-CoA thioesterase; CTE-II, 110 kDa cytosolic long-chain acyl-CoA thioesterase; DEHP, di(2-ethylhexyl)phthalate; MTE-I, 45 kDa mitochondrial very-long-chain acyl-CoA thioesterase; PTE-I, 47 kDa peroxisomal long-chain acyl-CoA thioesterase.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers Y09333 and Y09334.

purchased from Wako Pure Chemicals (Tokyo, Japan) or Sigma (St. Louis, MO, U.S.A.).

Purification of isoenzymes by using an immunoaffinity column with covalently bound anti-MTE-I antibodies

Anti-MTE-I antibodies [14] were coupled to 10 ml of cyanogen bromide-activated Sepharose (Pharmacia Biotechnology, Uppsala, Sweden) in accordance with the manufacturer's instructions. Mitochondrial matrix fractions, light mitochondrial fractions and 100000 ***g*** supernatants were prepared as described previously [6]. The immunoaffinity column was equilibrated with PBS. Aliquots of the respective subcellular fractions, containing enough protein to saturate the binding to the anti-MTE-I affinity column, were diluted 1:1 in PBS and then applied to the column, which was then washed with two column volumes of PBS followed by three column volumes of PBS containing 1 M NaCl. The adsorbed protein was eluted with 30 mM glycine, pH 2.8, and immediately neutralized by the addition of 1 M Tris, pH 8.0, to a final concentration of 50 mM. The eluted proteins were precipitated with $(NH_4)_2SO_4$ (70 % satn.) and dissolved in a minimal volume of 10 mM potassium phosphate buffer, pH 7.0, containing 0.5 % SDS. The purity of the enzyme preparations was analysed by SDS/PAGE.

Digestion of the purified enzymes with Protease V8

Aliquots of the purified enzyme preparations were subjected to SDS/PAGE [10 % (w/v) gel] and the band corresponding to the respective enzyme was cut out, washed and dried for Protease V8 digestion in SDS/PAGE gels by the method of Cleveland et al. [16]. The dried gel slices were placed in the wells of a 4 % (w/v) stacking gel and left to swell before the protease was added at indicated concentrations. The separation of the resulting peptides was performed in a 12.5 % (w/v) resolving gel.

Peptide sequencing

Immunopurified and digested enzymes were electroblotted to a PVDF membrane (Bio-Rad). The blot was stained with Amido Black, the bands were cut out and the peptide sequences of the major stained bands were determined with a model 477A gas-phase sequencer (Applied Biosystems) connected to an in-line model 120A phenylthiohydantoin analyser (Applied Biosystems).

Cloning of cDNA species

Plaques (8×10^5) of a λgt11 cDNA library prepared from clofibrate-induced male rat liver [17] were screened with an oligonucleotide probe corresponding to a sequenced proteolytic fragment, LEVLDGHEPDG, of purified MTE-I. A 33-mer oligonucleotide probe was synthesized based on the identity of the MTE-I derived peptide with a translated mouse-expressed sequence tag (GenBank accession number L11811) [18]. After 20 positive clones had been obtained, the phage DNA was prepared [19] and the inserts were digested with *Eco*RI and subcloned into pBluescript II SK$^+$ (Stratagene, La Jolla, CA, U.S.A.).

Each clone was sequenced 350–400 bp from each end with SK and KS primers (Stratagene); alignment of these sequences showed that cDNA species corresponding to two different mRNA species had been isolated. The longest cDNA species, corresponding to MTE-I and CTE-I respectively, were completely sequenced on both strands by using serial deletions with appropriate restriction enzymes, followed by subcloning and sequencing of the new ends with SK and KS primers. The DNA sequencing was performed by the dideoxy sequencing method [20] with a Dye Terminator cycle sequencing kit (Perkin–Elmer, Hilden, Germany) and 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.) for 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 [21]. RNA (5 μg) from each tissue 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. The cDNA probe was prepared by digestion of the full-length MTE-I cDNA clone by *Sma*I and purification of the 242 bp fragment, which was labelled with ^{32}P (Megalabel kit®, Takara). The hybridized bands were detected in a BAS-1500 system (Fuji Film Co., Minamiashigara, Japan).

Expression of MTE-I cDNA in Chinese hamster ovary (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*RI site of the pCXN2 mammalian expression vector [22]. A 10 μg sample of the plasmid was transfected into CHO cells with a calcium phosphate co-precipitation method as described previously [23]. To obtain stable transfectants, the cells were cultured with the addition of 0.5 mg/ml Geneticin disulphate (Wako Pure Chemicals). After harvesting, the cells were washed with 0.5 ml of PBS and then sonicated. After centrifugation the supernatant was analysed by Western blotting to verify expression from the introduced cDNA.

Immunoprecipitation of endogenous CHO cell acyl-CoA thioesterase activity

CHO cell lysates were prepared as described above. A 50 μl aliquot of the lysate was incubated for 1 h at 25 °C with 5 μl of an antibody raised against CTE-II [2]. After centrifugation the enzyme activity was measured in the resulting supernatant [13].

Immunofluorescence microscopy

CHO cells were cultured in 1 ml of Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum on cover slips in 8 cm^2 dishes. The cells were fixed with 4 % (w/v) paraformaldehyde for 15 min. After being washed with PBS the cells were solubilized with 0.1 % (v/v) Triton X-100 in PBS for 1 h, followed by blocking for 2 h with 10 % fetal bovine serum in PBS containing 0.1 % Triton X-100. The cells were then incubated overnight with anti-MTE-I antibodies diluted in PBS containing 10 % fetal bovine serum and 0.1 % Triton X-100. After four washes with PBS containing 0.1 % Triton X-100, the cells were incubated with FITC-conjugated anti-(rabbit IgG) antibodies for 1 h. The cells were then analysed by fluorescence microscopy.

Immunoelectron microscopy

CHO cells were isolated and fixed for 2 h in 4 % (w/v) paraformaldehyde/0.1 % glutaraldehyde/0.1 M sodium phosphate (pH 7.4). The subsequent procedures for immunolabelling with

anti-(MTE-I) and analysis by electron microscopy have been described previously [24].

Other methods

Acyl-CoA thioesterase activity was routinely followed spectrophotometrically with 5,5′-dithiobis-(2-nitrobenzoic acid), as described previously [14]. The appropriate acyl-CoA was preincubated and the reaction was started by the addition of enzyme. Protein was determined by using the Coomassie protein assay reagent (Pierce) with BSA as a standard.

RESULTS

Purification of MTE-I isoenzymes from different subcellular fractions

We have shown previously that isoenzymes of MTE-I, which are recognized by antibodies raised against MTE-I [14], are present in the cytosol and peroxisomes [6]. To compare the peptide maps after Protease V8 digestion, and to obtain peptide sequence data from the different isoenzymes, we established an immunoaffinity chromatography procedure to purify the different isoenzymes. The isolated isoenzymes showed slightly different mobilities on SDS/PAGE. PTE-I and MTE-I migrated as 47 and 45 kDa proteins respectively, and CTE-I showed an intermediate molecular mass of approx. 46 kDa. After digestion of the proteins with Protease V8, the peptide pattern derived from MTE-I was easily distinguished from those of CTE-I and PTE-I (Figure 1). However, no clear difference was seen between CTE-I and PTE-I.

Isolation and nucleotide sequences of cDNA species encoding MTE-I and CTE-I

To isolate clones corresponding to both MTE-I and CTE-I we synthesized an oligonucleotide, as described in the Experimental section, which was expected to recognize both types of cDNA because only one amino acid residue differed in this region of the two corresponding sequenced peptides. The screening resulted in the isolation of 20 positive clones. Of these, four corresponded to MTE-I and sixteen to CTE-I. The longest MTE-I clone was

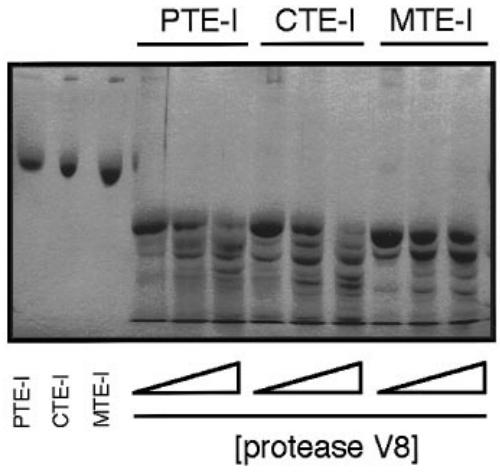

Figure 1 Digestion of immunopurified isoenzymes with Protease V8

PTE-I, CTE-I and MTE-I were purified on immobilized anti-MTE-I as described in the Experimental section. The isolated isoenzymes were electrophoresed in 12.5% (w/v) acrylamide gels before (left three lanes) and after digestion with Protease V8. The digestions were performed with increasing amounts of Protease V8 (10, 50 and 100 ng).

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       CGGGCCTACGGCTCAGTCTAAGGACTGCAAATAGGCAGCTGGCCACTAGAGGATCTCTAA    60
       CTTTTCCTACGAAACTGAGGGCTGAAGTCAAAGATACAAAATGGTGGCCTCGTCTTTCGC   120
                                                   M  V  A  S  S  F  A
       TGTCCTGAGAGCAAGCAGGTTGTGCCAATGGGGTTGGAAGAGCTGGACGCAGCTGTCAGG   180
    8   V  L  R  A  S  R  L  C  Q  W  G  W  K  S  W  T  Q  L  S  G
       TCCTCCGCCGCTCAGCACCGGTGGCCGGACCACTTTTGCGCGGACAAATGCTACGCTGAG   240
   28   P  P  P  L  S  T  G  G  R  T  T  F  A  R  T  N  A  T  L  S
       CCTGGAGCCCGGGAGCCGCAGCTGCTGGGACGAGCCGTTGAGCATCACCGTGCGCGGCCT   300
   48   L  E  P  G  S  R  S  C  W  D  E  P  L  S  I  T  V  R  G  L
       GGCCCCCGAGCAGCCCGTCACGCTGCGCGCGGCCCTGCGTGACGAGAAGGGCGCGCTCTT   360
   68   A  P  E  Q  P  V  T  L  R  A  A  L  R  D  E  K  G  A  L  F
       CCGAGCCCACGCGCGCTACCGCGCCGACGCCGGTGGTGAGCTGGACCTGGCGCGCGCTCC   420
   88   R  A  H  A  R  Y  R  A  D  A  G  G  E  L  D  L  A  R  A  P
       CGCGCTGGGCGGCAGCTTCACGGGGCTCGAGCCCATGGGGCTGATCTGGGCCATGGAGCC   480
  108   A  L  G  G  S  F  T  G  L  E  P  M  G  L  I  W  A  M  E  P
       CGAACGGCCTCTCTGGCGCCTGGTCAAGCGCGACGTGCAGAAGCCTTATGTGGTGGAGCT   540
  128   E  R  P  L  W  R  L  V  K  R  D  V  Q  K  P  Y  V  V  E  L
       GGAGGTGCTGGACGGACACGAGCCCGACGGCGGTCAGCGGCTGGCACAGGCAGTGCACGA   600
  148   E  V  L  D  G  H  E  P  D  G  G  Q  R  L  A  Q  A  V  H  E
       GCGTCACTTCATGGCTCCAGGGGTGCGGCGCGTGCCCGTGCGCGACGGGCGGGTGCGCGC   660
  168   R  H  F  M  A  P  G  V  R  R  V  P  V  R  D  G  R  V  R  A
       CACGCTCTTCCTGCCCCCAGAACCTGGGCCCTTTCCTGAAATCATAGACCTTTTTGGAGT   720
  188   T  L  F  L  P  P  E  P  G  P  F  P  E  I  I  D  L  F  G  V
       TGGAGGCGGCCTTCTGGAGTACCGGGCGAGTCTGCTGGCTGGGAAGGGTTTTGCCGTCAT   780
  208   G  G  G  L  L  E  Y  R  A  S  L  L  A  G  K  G  F  A  V  M
       GGCTCTGGCTTATTACAACTACGACGACCTCCCCAAGACCATGGAAACCATGCGCATTGA   840
  228   A  L  A  Y  Y  N  Y  D  D  L  P  K  T  M  E  T  M  R  I  E
       GTACTTTGAAGAAGCCGTGAACTACCTGCGTGGCCACCCTGAGGTAAAAGGACCAGGAAT   900
  248   Y  F  E  E  A  V  N  Y  L  R  G  H  P  E  V  K  G  P  G  I
       TGGGCTGCTTGGGATTTCCAAAGGGGGTGAACTTGGCCTTGCTATGGCCTCCTTCCTGAA   960
  268   G  L  L  G  I  S  K  G  G  E  L  G  L  A  M  A  S  F  L  K
       GGGCATCACGGCTGCTGTTGTCATCAATGGCTCCGTGGCTGCTGTTGGGAACACCGTATG  1020
  288   G  I  T  A  A  V  V  I  N  G  S  V  A  A  V  G  N  T  V  C
       CTACAAGGATGAGACTATACCCCCTGTGTCCCTTCTGAGAGACAAAGTCAAAATGACCAA  1080
  308   Y  K  D  E  T  I  P  P  V  S  L  L  R  D  K  V  K  M  T  K
       AGATGGTCTCTTGGATGTCGTGGAAGCTCTGCAAAGCCCTTTGGTAGACAAGAAGAGCTT  1140
  328   D  G  L  L  D  V  V  E  A  L  Q  S  P  L  V  D  K  K  S  F
       CATCCCTGTGGAAAGGTCTGACACGACCTTCCTGTTCCTCGTTGGTCAGGATGACCACAA  1200
  348   I  P  V  E  R  S  D  T  T  F  L  F  L  V  G  Q  D  D  H  N
       CTGGAAGAGCGAGTTCTATGCCAGAGAGGCCTCCAAACGCTTGCAGGCCCACGGGAAAGA  1260
  368   W  K  S  E  F  Y  A  R  E  A  S  K  R  L  Q  A  H  G  K  E
       GAAGCCCCAGATCATCTGCTACCCAGAAGCAGGGCACTATATCGAGCCTCCTTACTTCCC  1320
  388   K  P  Q  I  I  C  Y  P  E  A  G  H  Y  I  E  P  P  Y  F  P
       ACTGTGCAGCGCTGGCATGCACCTCTTGGTGGGTGCTAACATCACCTTTGGAGGGGAGCC  1380
  408   L  C  S  A  G  M  H  L  L  V  G  A  N  I  T  F  G  G  E  P
       TAAGCCTCACTCTGTGGCCCAGTTGGATGCATGGCAGCAACTCCAGACTTTCTTCCACAA  1440
  428   K  P  H  S  V  A  Q  L  D  A  W  Q  Q  L  Q  T  F  F  H  K
       ACAGTTGAGTGGTAAGAGTTAGGAGGTGCCCCCTAAAATATAACCTGTTATGTGGTGGTT  1500
  448   Q  L  S  G  K  S  *
       TGGGGAAAAACCCAAATATCAGAATGCCACTTCAGTTTAGTTCATTTGAACACATACTAA  1560
       TTTTTTTAAGTTTCTTTCTTCCTTCCTTTCTTTCTTTCTGTTTTTTTTTTTTTGTTGTTGT  1620
       TGTTGTTGTTTGTTTGTTTGTTTGAGACAGGGTTTGTCTGTTTACCCCTGGCTGGCCTGG  1680
       AACTTGCTTTGTAGACCAGAGGCTAGGCCTG                                1711
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Figure 2 Nucleotide and deduced amino acid sequences

Nucleotide sequence and deduced amino acid sequence of MTE-I. The stop codon is identified by an asterisk. The underlined amino acid sequences correspond to the peptide sequences obtained from proteolytically digested MTE-I.

considered to contain the complete open reading frame for MTE-I (Figure 2). The nucleotide sequence for MTE-I encompasses 1711 bp, including 100 bp in the 5′ non-coding region, followed by a coding region of 1359 bp and 252 bp of non-coding sequence at the 3′ end. We were unable to isolate a full-length clone encoding CTE-I. The longest clone of 1561 bp lacked 21 bp towards the 5′ end. Another clone of 1050 bp extended further 30 bp in the 5′ direction and the 1020 bp overlap showed 100% identity. The two overlapping clones contained the complete amino acid coding region. The assembled nucleotide sequence for CTE-I encompasses 1591 bp, including 9 bp in the 5′ non-coding region, followed by a coding region of 1257 bp and 325 bp in the 3′ non-coding region. The mouse homologue to CTE-I has been cloned and characterized further (P. J. G. Lindquist, L. T. Svensson and S. E. H. Alexson, unpublished work).

The largest peptides isolated from the digests of MTE-I and CTE-I were sequenced. The sequence obtained from the peptide derived from MTE-I was LEVLDGHEPDGGQRLAQAVH and the sequence of the corresponding peptide from CTE-I was LEVLDGHKPDGGRLLAR. Thus the two peptide sequences were different in four residues out of seventeen. The sequence RHFMAPGVRRV was obtained from another peptide of the digested MTE-I. The peptide sequences obtained from purified MTE-I matched the deduced amino acid sequence of the corresponding cDNA exactly (Figure 2, underline).

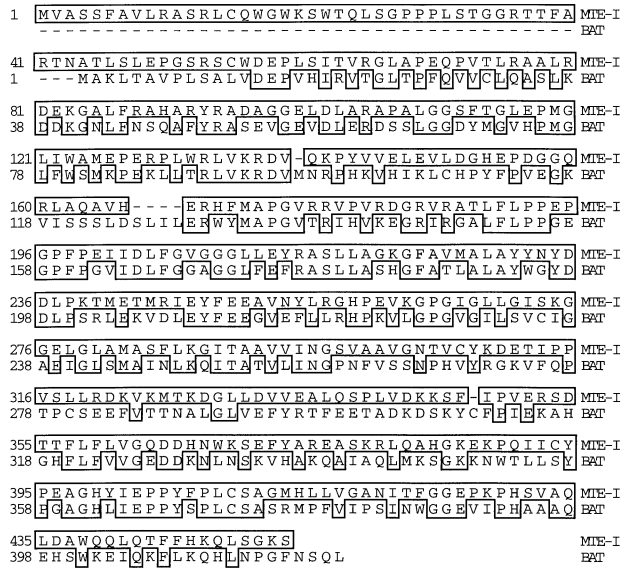

Figure 3 Amino acid sequence alignment of MTE-I and BAT

The deduced amino acid sequences of MTE-I and rat liver BAT are aligned and the residues identical with those in MTE-I are boxed.

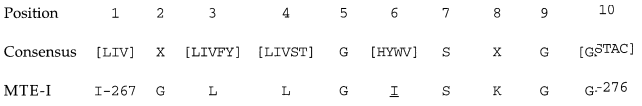

Figure 4 Comparison of residues 267–276 in the MTE-I sequence with the lipase active-site serine motif

The alternatives in the consensus sequence from the Prosite sequence motif database are presented and aligned with the putative active site of MTE-I. The residue that does not fit into the consensus sequence is underlined (I).

Analysis of the deduced amino acid sequence and homology search

Translation of the open reading frame of the cloned MTE-I cDNA resulted in a polypeptide of 453 residues with a calculated molecular mass of 49707 Da, which is larger than that of the purified MTE-I protein (45 kDa). Analysis of the deduced amino acid sequence by the computer program PSORT [25] suggested a mitochondrial localization. As mitochondrial matrix proteins often contain a leader peptide (usually of 20–80 residues) we examined the properties of the N-terminal end of the translation product in relation to suggested criteria for mitochondrial leader peptides [26,27]. Acidic amino acids are not allowed in mitochondrial leader peptides and the first acidic residue in the MTE-I sequence is Glu-49. Because the mature MTE-I was N-terminally blocked we have no information about the first residues of the mature enzyme. It has been shown that the mitochondrial processing peptidase prefers an arginine residue at position -2 relative to the cleavage site [28]. Analysis of the putative leader sequence suggests that processing of the leader peptide occurs between Thr-42 and Asn-43. When this N-terminal peptide is plotted as a helical wheel it forms a positively charged amphiphilic structure with four arginine residues and one lysine. The mature enzyme would have a calculated molecular mass of 45127 Da, which is in close agreement with the experimental value of 45 kDa. The position of the suggested cleavage site is supported by a comparison of the amino acid sequences of MTE-I and CTE-I, which show that the putative initiator methionine of CTE-I aligns with Thr-42 of MTE-I (results not shown). This indicates that the N-termini of the mature MTE-I and CTE-I are essentially identical.

An extensive search in DNA sequence databases showed a high degree of overall sequence similarity only to human and rat bile acid-CoA:amino acid *N*-acyltransferase (BAT, EC 2.3.1.65) [29,30]. The MTE-I amino acid sequence was approx. 40% identical with that of rat BAT (Figure 3). A cysteine residue has been implicated in the formation of an *S*-ester substrate intermediate during the acyl transfer reaction of BAT [29,31,32], and only three cysteine residues are conserved between rat and human BAT (results not shown). One of these cysteine residues coincides with the putative active site Ser-273 in MTE-I (Figure 3), which suggests a structural conservation and functional modification during the evolution of the active-site region between the two different enzymes.

In the deduced MTE-I amino acid sequence we found the typical lipase active-site serine sequence motif (Figure 4), corresponding to Ser-273 of MTE-I. In lipases and serine esterases a histidine residue participates in the catalytic mechanism by catalysing the hydrolysis after nucleophilic attack by the active-site serine. In MTE-I, His-414 is present within a Gly-Xaa-His motif, which has been shown to be necessary for the hydrolytic activity of other thioesterases [33,34]. No other significant structural motifs were found. Searches in expressed sequence tag databases, with the nucleotide sequence corresponding to the open reading frame of MTE-I, revealed high similarities to multiple entries generated from human tissues.

Effect of DEHP treatment on levels and tissue-specific expression of mRNA

In a previous study we did not detect distinct signals by Western blot analysis that discriminated between MTE-I and CTE-I [14]. However, by improving the conditions for separation by SDS/PAGE in the present study we were able to examine the expression and regulation of the respective proteins. In addition, the detection of MTE-I-specific mRNA with cDNA clones as probes were hampered by the high degree of sequence identity with CTE-I. The full-length cDNA clone hybridized to three mRNA species of different sizes (1.8, 2.6 and approx. 3.5 kb) on Northern blots (results not shown). To detect the specific MTE-I mRNA, a cDNA probe corresponding to the 5′ end of the open reading frame encoding the putative mitochondrial leader sequence was constructed as described in the Experimental section. This probe hybridized only to a single mRNA species of approx. 2.6 kb. Analysis by Western and Northern blotting of liver samples from control rats showed that under normal conditions MTE-I could hardly be detected either at the protein level or at the mRNA level (Figure 5A). As early as 1 day after initiation of feeding with DEHP, MTE-I protein was clearly detected and the corresponding mRNA signal was at maximal level. However, the signal for MTE-I mRNA was transiently increased and was decreased after 14 days compared with the maximal level. The protein reached maximal level after 4 days and remained high throughout the 14 days of the treatment. Also, after 2 days of the treatment, two additional proteins of higher molecular masses were detected, corresponding to CTE-I and possibly PTE-I. Interestingly, the signal corresponding to CTE-I protein was induced more slowly relative to the signal for MTE-I protein.

Various tissues from control rats and rats treated with DEHP for 14 days were examined for expression of CTE-I and MTE-I

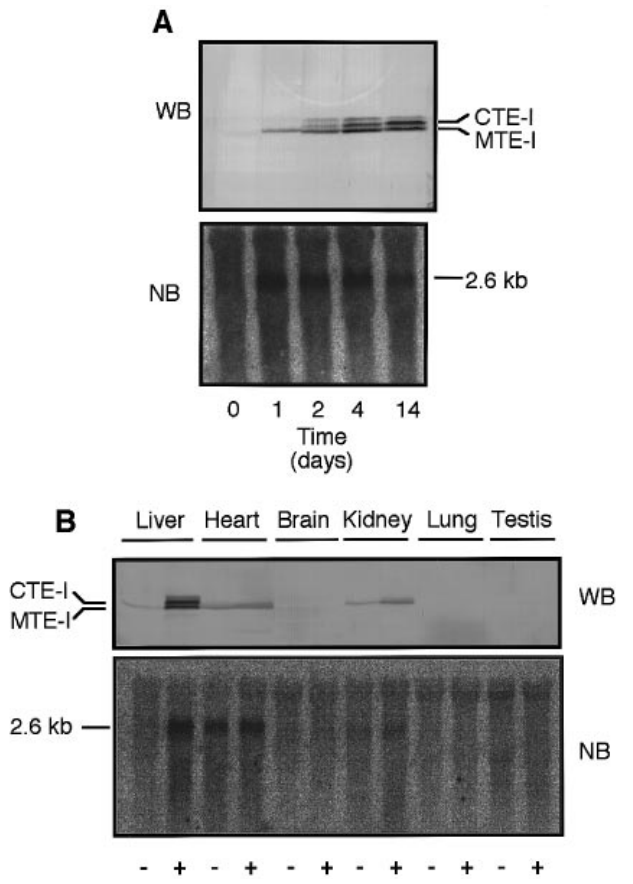

Figure 5 Analysis of the effect of treatment with DEHP on the liver expression and tissue specificity of MTE-I and CTE-I

(**A**) Liver homogenates (10 μg) and total liver RNA (5μg) 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, Northern blot analysis (NB). Each blot is representative of three different 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 by Northern blotting (lower panel). Each blot is representative of three different experiments.

protein, and MTE-I mRNA (Figure 5B). It was concluded that MTE-I is induced strongly in the liver, expressed constitutively in heart and induced only moderately by the treatment in kidney. CTE-I protein was induced strongly in liver but weakly in kidney.

Expression of MTE-I cDNA in CHO cells

To verify that the MTE-I cDNA encodes an acyl-CoA thioesterase protein and that the expressed protein is properly targeted to mitochondria and processed accordingly, we established stably transfected CHO cell lines. The selected cell lines were analysed for acyl-CoA thioesterase activity and for the presence of immunoreactive protein by Western blot analysis. Comparison of the mobilities of the labelled bands with purified MTE-I indicates that expressed MTE-I cDNA is translated properly and processed to its mature size, corresponding to 45 kDa (results not shown). Control CHO cells have high endogenous acyl-CoA thioesterase activities, approx. 100 nmol/min per mg of cell protein. We have shown previously that this endogenous activity emanates mainly from an enzyme that is

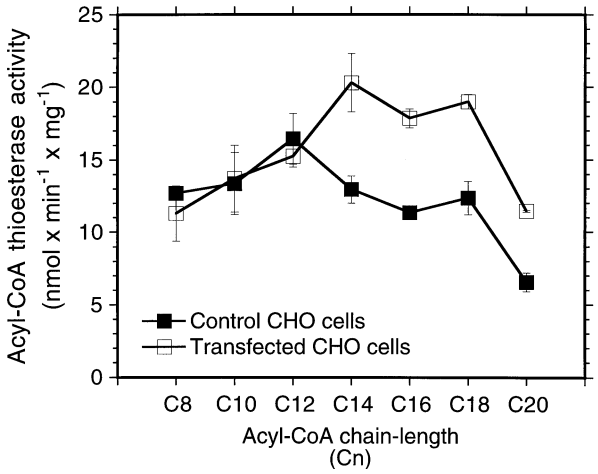

Figure 6 Expression of recombinant MTE-I in CHO cells

Control CHO cells and transfected cells expressing MTE-I were analysed for acyl-CoA chain length specificity at a substrate concentration of 10 μM. Each point represents the mean activity $\pm$ S.E.M. for three different culture dishes.

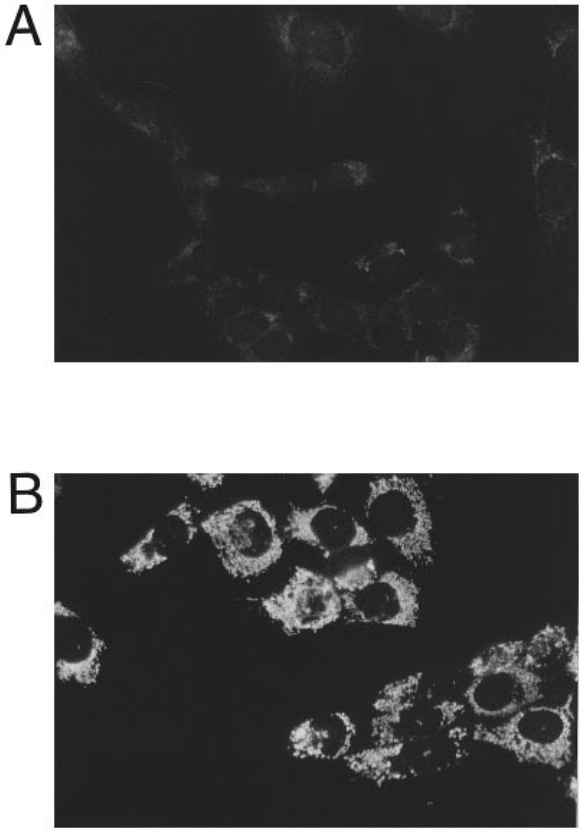

Figure 7 Immunofluorescence microscopy

(**A**) Representative views of control CHO cells; (**B**) CHO cells transfected with MTE-I cDNA. The cells were labelled with anti-MTE-I antibodies as described in the Experimental section. Magnification $\times$ 600.

immunologically cross-reactive with the peroxisome proliferator-induced cytosolic acyl-CoA thioesterase type II (CTE-II) [13]. To measure the expressed MTE-I we immunoprecipitated the CTE-

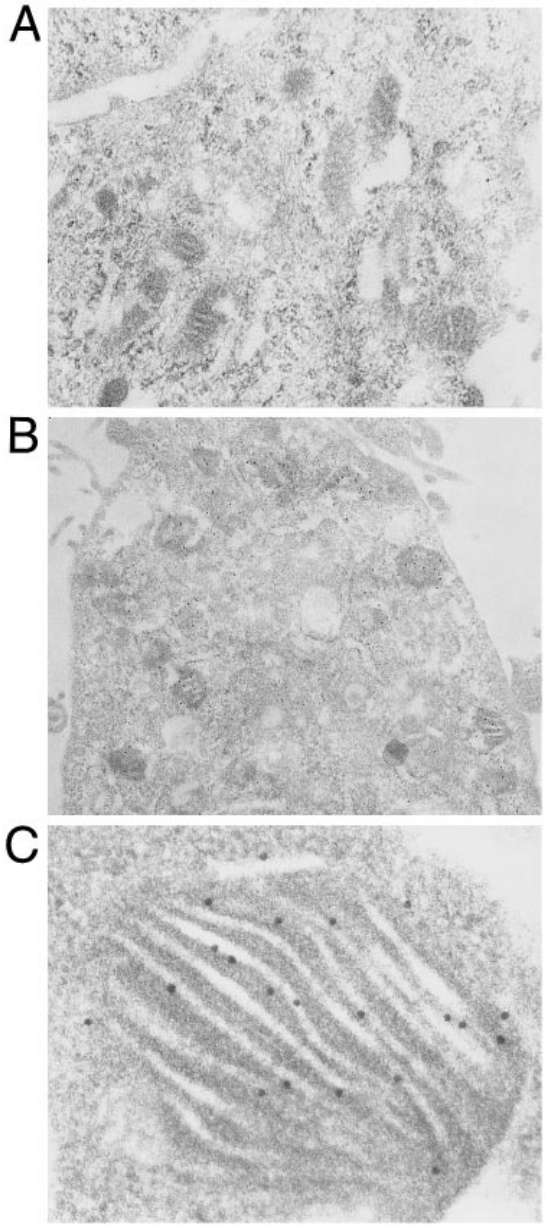

Figure 8 Immunoelectron microscopy of CHO cells labelled with antibodies to MTE-I

(**A**) Overview of control CHO cells (magnification × 23000); (**B**) overview of transfected CHO cells (magnification × 13000); (**C**) higher magnification of transfected CHO cells (magnification × 79000).

II-dependent activity before the measurement. Figure 6 shows the substrate specificities of control CHO cells and of one of the transfected CHO cell lines after immunoprecipitation. The transfected cells contained clearly higher acyl-CoA thioesterase activities with acyl-CoAs of 14–20 carbon atoms, in accordance with the substrate specificity of native MTE-I [14]. Next we examined the intracellular targeting of expressed MTE-I in CHO cells by immunofluorescence microscopy (Figure 7) and immunogold electron microscopy (Figure 8). Labelling of control CHO cells with antibodies against MTE-I showed only diffuse faint fluorescence (Figure 7A). The transfected cells, however, were labelled strongly with a punctate pattern, indicating an organellar targeting of the expressed MTE-I (Figure 7B). To further verify the subcellular localization of the expressed MTE-I, control and transfected CHO cells were analysed by immunoelectron microscopy. In the control CHO cells, only few gold particles were found and were not associated with any distinct cellular compartment (Figure 8A). In transfected cells, most gold particles were found over structures morphologically identified as mitochondria (Figure 8B). At high magnification it was obvious that most of the staining was associated with the matrix face of mitochondrial cristae (Figure 8C).

DISCUSSION

From previous studies it is clear that the induction of acyl-CoA thioesterase activity in rat liver by peroxisome proliferators is due to the induction of several enzymes present in the cytosol, mitochondria and peroxisomes [2,3,6,35]. In cytosol at least two enzymes are induced, here called CTE-I and CTE-II. CTE-I corresponds to ACH2 isolated by Yamada et al. [12]; CTE-II corresponds to the enzyme isolated by Miyazawa et al. [2] and to ACH1 later isolated by Yamada et al. [12]. Also in mitochondria at least two enzymes, tentatively named MTE-I and MTE-II, are strongly induced. MTE-I was recently isolated, and MTE-II was partly purified by us [14]. On the basis of immunological cross-reactivity between MTE-I and CTE-I it was concluded that they represent highly homologous isoenzymes, in spite of the different subcellular localizations. As antibodies raised against CTE-II immunoprecipitated approx. 50% of the acyl-CoA thioesterase activity in mitochondria [2], it is reasonable to assume that MTE-II is structurally related to CTE-II [14]. Taken together, these results therefore suggest that there exist two families of structurally related acyl-CoA thioesterases, which are induced by peroxisome proliferators in rat liver. Recently the cloning of the cDNA corresponding to CTE-II was described [13]. This enzyme was found to be highly homologous to another recently isolated and cloned brain acyl-CoA thioesterase [36,37].

In the present study we describe the cDNA cloning of MTE-I and CTE-I from rat liver. The cDNA species were isolated with an oligonucleotide probe that was synthesized on the basis of an amino acid sequence obtained from immunoaffinity-purified MTE-I. The identity of a cDNA corresponding to MTE-I was verified by complete matches of two peptide sequences derived from purified MTE-I and from the functional expression of the cDNA in CHO cells. In addition, on the basis of sequence comparison and computer modelling the deduced amino acid sequence of MTE-I contains a putative mitochondrial leader sequence. Western blot analysis, immunofluorescence and immunoelectron microscopy of transfected CHO cells showed that the expressed thioesterase was properly targeted to mitochondria and processed to the mature size.

A second cDNA was isolated and was highly similar to the MTE-I cDNA. However, the open reading frame encoded 419 amino acid residues. This cDNA was concluded to encode CTE-I because one peptide sequence derived from purified and digested CTE-I completely matched the corresponding deduced amino acid sequence. Alignment of the deduced amino acid sequences showed 92.5% identity. The major difference was that the sequence of CTE-I started at position 42 of the MTE-I sequence, which corresponds to the cleavage site of the putative mitochondrial leader sequence of MTE-I. In addition, the sequence of CTE-I extended seven residues longer at the C-terminal end, which explains the size difference observed on SDS/PAGE.

MTE-I contains a putative active-site region that is very similar to a general lipase active-site motif. The essential nucleophilic residue serine is present (residue 273 of MTE-I) and also

the hydrophobic properties needed for substrate binding. Several esterases containing a Gly-Xaa-Ser-Xaa-Gly active-site motif, which are active on water-soluble substrates, are inhibited by so-called serine esterase inhibitors, e.g. di-isopropyl fluorophosphate or PMSF. Although Ser-273 of MTE-I is the likely active site residue, the activity was not inhibited by serine esterase inhibitors [14]. However, the thiol-reactive agent *p*-chloromercuribenzoate inhibits MTE-I and CTE-I [12,14]. This suggests that also cysteine residues might be of importance for the catalytic activity. These surprising effects of active-site inhibitors are rather different from those of thioesterase I and thioesterase II of the fatty acid synthase systems, for example [38,39], but are similar to the behaviours of palmitoyl-protein thioesterase [34] and CTE-II [13].

Sequence comparisons in databases revealed high similarity to a number of expressed sequence tag sequences. However, the only apparent sequence similarity to known proteins was found with human and rat BAT (approx. 40% sequence identity). Interestingly, BAT contains a motif similar to the active site of lipases but with one very important difference: the putative active-site serine residue is replaced by a cysteine in BAT. In addition, a histidine residue (His-414 in MTE-I) present in the Gly-Xaa-His motif characteristic of thioesterases [34,40] is lacking from BAT. These differences in putative active-site amino acids might therefore explain the different kinetic mechanisms of the thioesterase compared with transferase activities of these enzymes.

Several papers have described molecular cloning of enzymes that are capable of hydrolysis of long-chain acyl-CoAs (see, for example, [13,34,37]). However, the physiological functions of most acyl-CoA thioesterases remain unclear. So far we have concentrated our studies on acyl-CoA thioesterases that are induced in rat liver by peroxisome proliferators. The reason for this is that their unclear physiological functions might be related to the metabolic changes in liver that are provoked by the administration of peroxisome proliferators to the rat. Further understanding of possible physiological functions might be obtained from studies on tissue expression and regulation. From Northern blot analysis, with a partial cDNA probe corresponding to the putative mitochondrial leader peptide of MTE-I, it was concluded that MTE-I is constitutively expressed in heart, whereas it is present in liver only after treatment with peroxisome proliferator. The expression pattern of MTE-I (and CTE-I) is very different from that of CTE-II, which is expressed constitutively in brain and testis but not in heart [13]. On the basis of the tissue expression pattern of CTE-II, we suggested that the function of CTE-II is possibly related to synthesis of phospholipids or steroids [13]. Although peroxisome proliferator-induced type I and type II acyl-CoA thioesterases both hydrolyse acyl-CoA *in vitro*, the low sequence similarity and the different tissue expressions clearly indicate that type I and II isoforms have different physiological functions. On the basis of the present results we speculate that MTE-I is involved in the constitutive metabolism of acyl-CoA in heart mitochondria and an adaptive metabolism of acyl-CoA in liver mitochondria. The metabolic event that matches these criteria most closely is the β-oxidation of fatty acids, which has been shown to be increased in liver mitochondria after treatment with peroxisome proliferators. The effects of peroxisome proliferators have been shown to be exerted both at the level of total β-oxidation [41] and at the level of the rate-limiting enzymes [17,23]. Thus, when the activity of the mitochondrial β-oxidation is high, MTE-I might prevent both the sequestration of intra-mitochondrial CoASH and increases in the concentration of long-chain acylcarnitine. If acyl-CoA, transacylated from acylcarnitine by carnitine palmitoyltransferase II, were allowed to accumulate, it would lead to product inhibition of carnitine palmitoyltransferase II with a concomitant increase in the concentration of long-chain acylcarnitine. This situation could be deleterious to mitochondrial membrane structures, owing to the strong surface-active and membrane-lytic properties of palmitoylcarnitine [42]. This suggests a physiological function for MTE-I that involves modulation of the concentration of mitochondrial long-chain acyl-CoA under situations when the rate of β-oxidation of fatty acids is lower than the rate of transport of long-chain acyl-CoA into the mitochondria. This situation might occur under fasting conditions when fatty acids are mobilized from peripheral tissues and accumulate in the liver. The function of CTE-I is obviously different, on the basis of the completely different subcellular localization. As acyl-CoAs are synthesized at the cytosolic face of endoplasmatic reticulum, peroxisomes and mitochondria [43,44], the function of CTE-I might be associated with one or more of these events.

We thank Dr. M. Souri for valuable help, and S. Miyazawa for the peptide sequencing. This investigation was supported by 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 grants from the Swedish Natural Science Research Council and Magnus Bergvalls Stiftelse. 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.

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Received 30 June 1997/29 September 1997; accepted 29 September 1997