

IDENTIFICATION OF DI-(2-ETHYLHEXYL) PHTHALATE-INDUCED CARBOXYLESTERASE 1 IN C57BL/6 MOUSE LIVER MICROSOMES: PURIFICATION, CDNA CLONING, AND BACULOVIRUS-MEDIATED EXPRESSION

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ABSTRACT:

Several mouse carboxylesterase (CES) isozymes have been identified, but information about their roles in drug metabolism is limited. In this study, we purified and characterized a mouse CES1 isozyme that was induced by di-(2-ethylhexyl) phthalate. Purified mouse CES1 shared some biological characteristics with other CES isozymes, such as molecular weight of a subunit and isoelectronic point. In addition, purified mouse CES1 behaved as a trimer, a specific characteristic of CES1A subfamily isozymes. The purified enzyme possessed temocapril hydrolase activity, and it was found to contribute significantly to temocapril hydrolase activity in mouse liver microsomes. To identify the nucleotide sequences coding mouse CES1, antibody screening of a cDNA library was performed. The deduced amino acid sequence of the obtained cDNA, mCES1, exhibited striking similarity to those of CES1A

isozymes. When expressed in Sf9 cells, recombinant mCES1 showed hydrolytic activity toward temocapril, as did purified mouse CES1. Based on these results, together with the findings that recombinant mouse CES1 had the same molecular weight of a subunit, the same isoelectronic point, and the same native protein mass as those of purified mouse CES1, it was concluded that mCES1 encoded mouse CES1. Furthermore, tissue expression profiles of mCES1 were found to be very similar to those of the human CES1 isozyme. This finding, together with our other results, suggests that mCES1 shares many biological properties with the human CES1 isozyme. The present study has provided useful information for study of metabolism and disposition of ester-prodrugs as well as ester-drugs.

Several prodrug strategies have been developed to enable drugs to exhibit optimal pharmacokinetics and pharmacological actions, and an esterification strategy is widely used to increase transcellular absorption of poorly permeable drugs administered orally. The requirements for a better ester-prodrug are that it is stable to hydrolytic breakdown in its absorptive stage and that it is easily hydrolyzed to generate an active compound once it enters the systemic circulation (Beaumont et al., 2003). Carboxylesterases (CESs, EC.3.1.1.1.) are essential to achieve these requirements because they play an important role in biotransformation of many ester-prodrugs.

Mammalian CESs are members of an α , β -hydrolase-fold family and are found in various mammals (Satoh and Hosokawa, 1998; Satoh et al., 2002). The expression of CESs is ubiquitous, with high levels in the liver, small intestine, kidney, and lung. CESs show such a broad range of substrate specificity that they can be involved in detoxification or biotransformation of many kinds of drugs as well as endoge-

nous fatty acid esters. It has been suggested that CESs can be classified into four major groups according to the homology of the amino acid sequence (Satoh and Hosokawa, 1998), and the majority of CESs that have been identified belong to the CES1 or CES2 family. Recent studies have shown that there are some differences between these families in terms of substrate specificity, tissue distribution, immunological properties, and gene regulation (Satoh and Hosokawa, 1998). For example, the preferential substrates for hCE-1 (also called hCE or CES HU1) (Kroetz et al., 1993; Satoh and Hosokawa, 1998), a human CES1 family isozyme, are thought to be compounds esterified by a small alcohol molecule, whereas those for hCE-2, a human CES2 family isozyme, are thought to be compounds esterified by a relatively large alcohol molecule (Pindel et al., 1997; Takai et al., 1997; Zhang et al., 1999; Satoh et al., 2002). It has also been shown that striking species differences exist (Inoue et al., 1979; Hosokawa et al., 1990, 1994; Prueksaritanont et al., 1996; Zhu et al., 2000). For example, Inoue et al. (1979) showed that esterase activity in the dog intestine is very weak and produced no appreciable active band in a disc electrophoresis coupled with staining of esterase activity. On the other hand, esterase activities were observed in the intestines of other species (human, rat, mouse, guinea pig, and rabbit) and found to

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ABBREVIATIONS: CES, carboxylesterase; CES D1, dog carboxylesterase D1; CES ML1, mouse carboxylesterase ML1; CES ML2, mouse carboxylesterase ML2; CES RL1, rat carboxylesterase RL1; CES P1, porcine carboxylesterase P1; DEHP, di-(2-ethylhexyl) phthalate; Ms, microsomes; PAGE, polyacrylamide gel electrophoresis; PNPA, *p*-nitrophenylacetate; PNPester, *p*-nitrophenylester; PNPB, *p*-nitrophenylbutylate; PNPP, *p*-nitrophenylpropionate; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mTGH, mouse triacylglycerol hydrolase; PP, peroxisome proliferator; PPAR α , peroxisome proliferator-activated receptor.

produce a few active bands in the electrophoretic assay. Since pharmacokinetic and pharmacological data of ester-prodrugs obtained from preclinical experiments using various animals are generally used as references for human studies, it is important to clarify the biochemical properties of each CES isozyme such as substrate specificity, tissue distribution, and transcriptional regulation.

The mouse is one of the most widely used experimental animals in the process of development of a drug, and several mouse CES isozymes have been identified (Ovnic et al., 1991a,b; Aida et al., 1993; Satoh and Hosokawa, 1998; Dolinsky et al., 2001; Furihata et al., 2003; Xie et al., 2003). However, information on the involvement of mouse CESs in drug metabolism is limited. We have reported that exposure of C57BL/6 mice to di-(2-ethylhexyl) phthalate (DEHP), a peroxisome proliferator, in their diet resulted in a significant increase in the amount of CES protein concomitant with an increase in the level of hydrolytic activity toward xenobiotics in mouse liver microsomes (Hosokawa et al., 1994). We have also recently shown that one of the mouse CES isozymes induced by DEHP is mCES2/microsomal acylcarnitine hydrolase, a CES2 family isozyme (Furihata et al., 2003). Our immunochemical study also suggested that mouse CES1 isozymes were induced by DEHP treatment, but they remained to be identified.

The purpose of this study was to identify mouse CES1 isozymes induced by DEHP. Purification, cDNA cloning, and functional expression revealed that one of them is mCES1. Our data also showed that mCES1 contributes significantly to hydrolysis of temocapril, an ester-prodrug of an angiotensin-converting enzyme inhibitor, in mouse liver microsomes. Thus, we provided useful information for study of the metabolism and disposition of ester-prodrugs as well as ester-drugs.

Materials and Methods

Animals and Preparation of Solubilized Fraction of Mouse Liver Microsomes. Adult male C57BL/6 mice (Japan SLC Inc., Shizuoka, Japan), 8 weeks of age, were used in this study. The mice were fed a laboratory animal chow (CE-2; Japan Clea, Tokyo, Japan) with or without 2% (w/w) DEHP as described previously (Hosokawa et al., 1994). Each group consisted of three mice. The mice were sacrificed, and the livers were removed, weighed, and perfused with 1.15% KCl. Microsomes were isolated by differential centrifugation as described previously (Hosokawa et al., 1987) and were solubilized with 0.5% cholic acid in 10 mM Tris-HCl buffer (pH 8.0). The mixture was centrifuged at 10,000g for 30 min, and the supernatant was removed and used for subsequent experiments. Solubilized fractions of control and DEHP-treated mouse liver microsomes are referred to here as control Ms and DEHP Ms, respectively. Protein concentrations were determined by using a Bio-Rad Dc Protein Assay kit (Bio-Rad, Hercules, CA). All subsequent procedures were performed at 0–4°C.

Purification of Mouse CES1 from DEHP-Treated C57BL/6 Mouse Liver Microsomes. Purification of the CES isozyme that can hydrolyze temocapril from DEHP-treated C57BL/6 mouse liver was carried out essentially according to the procedure reported in our previous paper (Hosokawa et al., 1987). All steps were carried out at 4°C, and the peak fraction was detected by *p*-nitrophenylacetate (PNPA) hydrolase activity. Microsomes were solubilized in 100 mM Tris-HCl buffer, pH 8.0, containing 1% (w/v) saponin, at a final protein concentration of 2.7 mg/ml. The solution was stirred for 60 min at 4°C and then centrifuged at 105,000g for 60 min. The supernatant was fractionated by ammonium sulfate precipitation. The 30 to 70% (w/v) precipitation was resuspended in 10 mM Tris-HCl buffer (pH 8.0). The solution was gel-filtered on a Sephadex G-150 column (2.6 × 90 cm) (Amersham Biosciences Inc., Piscataway, NJ) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). Two peak fractions (high molecular weight and low molecular weight) were obtained. The high molecular weight fraction was applied to a column (3.0 × 8.0 cm) of Whatman DE-52 (Whatman, Maidstone, UK) that had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was washed with the same buffer and then eluted with a stepwise NaCl gradient in 10 mM

Tris-HCl buffer (pH 8.0). One peak fraction was collected by elution with 60 mM NaCl. The peak fraction was pooled and dialyzed for 24 h against three changes of 2 liters of 25 mM bis-Tris-HCl buffer (pH 6.5). The dialyzed fraction was applied to a column (1.0 × 30 cm) of chromatofocusing gel PBE 96 (Amersham Biosciences Inc.), equilibrated with 25 mM bis-Tris-HCl buffer (pH 6.5). The active fraction was eluted with 270 ml of Polybuffer 74 (Amersham Biosciences Inc.) and diluted at a ratio of 1:8. The pH of the fraction was adjusted to 4.5 with HCl. The active fraction was pooled and dialyzed for 24 h against two changes of 2 liters of 20 mM Tris-HCl buffer (pH 7.6) containing 0.1 M NaCl, 0.1 mM MgCl₂, and 0.5 mM CaCl₂. The dialyzed fraction was applied to a column (1.5 × 5.0 cm) of Con A-Sepharose (Amersham Biosciences Inc.) and equilibrated with 20 mM Tris-HCl buffer (pH 7.6) containing 0.1 M NaCl, 0.1 mM MgCl₂, and 0.5 mM CaCl₂. The column was washed with 20 mM Tris-HCl buffer (pH 7.6) containing 0.5 M NaCl, 0.1 mM MgCl₂, and 0.5 mM CaCl₂ and subsequently eluted with 20 mM Tris-HCl buffer (pH 7.6) containing 0.2 M α -methylmannoside, 0.1 M NaCl, 0.1 mM MgCl₂, and 0.5 mM CaCl₂. The fractions showing a single protein band in SDS-polyacrylamide gel electrophoresis (PAGE) were combined. The purified isozyme, termed mouse CES1, could be stored at –80°C for several months without loss of enzyme activity.

Preparation of Antibodies. Antibodies against purified CES P1, a porcine CES isozyme (Satoh et al., 1994), were raised in male Japanese white rabbits (2.5–3.0 kg in body weight) according to a previously described procedure (Hosokawa et al., 1987). Anti-CES RL1 antibodies and anti-CES D1 antibodies were prepared as previously described (Hosokawa et al., 1990, 2001). The IgG fraction of antiserum was purified from whole antibodies by using a HiTrap Protein A HP (Amersham Biosciences Inc.) and a PD-10 column (Amersham Biosciences Inc.) according to the manufacturer's protocol.

Determination of Hydrolase Activity. *p*-Nitrophenylesters (PNPesters) were obtained from the following sources: PNPA, *p*-nitrophenylbutylate (PNPB), *p*-nitrophenylvalerate, *p*-nitrophenylhexanoate, *p*-nitrophenyloctanoate, and *p*-nitrophenyllauroate were obtained from Nacalai Tesque (Kyoto, Japan), *p*-nitrophenylpropionate (PNPP) was obtained from Wako Pure Chemicals (Osaka, Japan), and *p*-nitrophenyldecanoate was obtained from Sigma-Aldrich (St. Louis, MO). Hydrolysis of PNPesters was determined in 50 mM Tris-HCl buffer (pH 8.0) at 30°C by colorimetrically measuring the amount of *p*-nitrophenol released according to the method of Krisch (1966). The substrate concentration of PNPA for kinetic study ranged from 10 to 600 μ M, and that of other *p*-nitrophenol esters for determination of hydrolytic activity was 50 μ M.

Butanilicaine was obtained from Hoechst AG (Frankfurt, Germany). Butanilicaine hydrolase activity was also determined by spectrophotometric analysis in 50 mM Tris-HCl buffer (pH 8.6), 0.225% cholic acid, and 9.0% glycerol as previously described (Hosokawa et al., 1990). The substrate concentration of butanilicaine for kinetic study ranged from 62.5 μ M to 1.5 mM.

Temocapril hydrolase activity was assayed in 50 mM HEPES buffer (pH 7.4), and temocaprilat, a metabolite of temocapril, was analyzed by using the following high-performance liquid chromatography method (Mori et al., 1999) with slight modifications. The high-performance liquid chromatography system consisted of a model L-6000 pump (Hitachi, Tokyo, Japan), a model L4000H UV detector (Hitachi), a model AS-2000 autosampler (Hitachi), a model D-2500 integrator (Hitachi), and a 4.6 × 150 mm YMC-Pack Ph A-402 column (YMC, Tokyo, Japan). The mobile phase consisted of 0.24% phosphoric acid/acetonitrile (68:32, v/v) and was delivered at a flow rate of 0.7 ml/min. Temocaprilat was detected at a wavelength of 233 nm. A calibration curve was generated from 0.5 to 100 μ M by processing the authentic standard substance through the entire procedure. Clofibrilic acid (Sigma-Aldrich) was used as an internal standard. Temocapril and temocaprilat were kindly supplied by Dr. Toshihiko Ikeda (Analytical and Metabolic Research Laboratories, Sankyo Co., Tokyo, Japan). The substrate concentration of temocapril for kinetic study ranged from 24 μ M to 300 μ M.

Specific activities of CES toward all substrates used were expressed in terms of the amount of substrate hydrolyzed in 1 min under specified conditions. Enzyme kinetic parameters were estimated using a computer program (DeltaGraph version 4.5; SPSS Inc., Chicago, IL) designed for nonlinear regression analysis as described previously (Takanashi et al., 2000). Each mean kinetic value was the average of three individual experiments with \pm S.D. Student's *t* test was performed to determine significance of difference between two groups. *P* values less than 0.05 were taken to be significant.

Inhibition Assay by Using Specific IgG. Inhibition of hydrolase activity by using a specific IgG was performed according to the procedure described previously (Hosokawa et al., 1994) with slight modification. Control Ms ($n = 3$) and DEHP Ms ($n = 3$) were incubated with control or specific IgG (either anti-CES D1 IgG or anti-CES RL1 IgG) (0.75 mg) for 30 min at 37°C, and the mixtures were left for 24 h at 4°C. Then, nProtein A Sepharose 4 Fast Flow (Amersham Biosciences Inc.) was added to the mixture. After incubation on ice for 1 h, the mixture was centrifuged for 10 min at 20,000g. The supernatant was removed and used for a determination of hydrolase activity as described above.

N-Terminal Sequences of Mouse CES1. N-terminal amino acid sequences of mouse CES1 were determined by using a Hitachi C-8500 automatic amino acid analyzer (Hitachi). Amino acids were sequenced by automated Edman degradation using a model 470A gas-phase sequencer (Applied Biosystems, Foster City, CA) with an on-line Spectra-Physics Model SP8100 PTH amino acid analyzer (Applied Biosystems) as described previously (Hosokawa et al., 1990).

Antibody Screening of cDNA Library. Antibody screening of a cDNA library was performed as described previously (Hosokawa et al., 2001). A ZAP cDNA library was constructed from DEHP-treated C57BL/6 mouse liver and was screened by anti-CES RL1 antibodies. Binding of this rabbit primary antibody to fusion proteins on nitrocellulose overlay was detected with peroxidase-conjugated goat anti-rabbit IgG (Amersham Bioscience). Binding of the second antibody was visualized with diaminobenzidine. cDNA fragments inserted into phage DNA of positive clones were isolated by EcoRI digestion and subcloned into the pBluescript SK (-) vector. The nucleotide sequences were determined using a Dye Terminator Cycle Sequencing-Quick Start Kit and CEQ 2000 DNA Analysis System (Beckman Coulter, Fullerton, CA) and were confirmed by sequencing at least twice in each direction. The cDNA clone obtained was named mCES1.

Baculovirus-Mediated Expression of mCES1 in Sf9 Cells. The recombinant mCES1 was expressed in Sf9 cells by using a BAC-TO-BAC Baculovirus Expression System (Invitrogen, Carlsbad, CA) in accordance with the directions of the manufacturer. The cDNA in the cloning vector was subcloned into the pFAST BAC1 vector using EcoRI and alkaline phosphatase. The pFAST BAC1 vector containing mCES1 was transformed into DH10Bac cells, and this was followed by transposition of the inserts into bacmid DNA. Likewise, nonrecombinant bacmid DNA (mock) was also prepared. The recombinant and mock bacmid DNAs were separately transfected into Sf9 cells with CellFECTIN reagent (Invitrogen), and the virus was harvested 72 h later. The cells were centrifuged at 1700g for 10 min to separate cells and virus. The supernatant containing the virus was stored at 4°C in the dark with 5% fetal bovine serum until used for infection. Cells were routinely infected with the virus and were harvested 72 h after infection, washed twice with phosphate-buffered saline, and stored at -80°C until used. Lysates were prepared by disrupting the cells with a sonicator until the cells were completely lysed as determined by microscopy. Cytosol of Sf9 cells expressing mCES1 and that of Sf9 cells infected with mock virus were prepared by subjecting the cell lysate to centrifugation (105,000g for 60 min at 4°C), and they were named mCES1/Sf9 and mock/Sf9, respectively.

Preparation of Mouse Tissue Homogenates and Esterase Activity Staining after Nondenaturing PAGE. Three 8-week-old C57BL/6 male mice (Japan SLC, Inc.) were sacrificed, and nine tissues (brain, thymus, lung, heart, liver, kidney, small intestine, epididymal adipose tissue, and testis) were removed from each mouse. Tissue samples of equal weight were pooled, and the homogenate was prepared using a Potter-Elvehjem homogenizer in 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris, pH 7.4 buffer at 4°C. The protein concentration was determined as described above. Esterase activity staining after nondenaturing PAGE was performed according to the method of Mentlein et al. (1980). α -Naphthylacetate was obtained from Tokyo Kasei (Tokyo, Japan). Different amounts of homogenate were used in this method to adjust band intensity: 20 μ g of brain, 15 μ g of thymus, 7 μ g of lung, 10 μ g of heart, 1 μ g of liver, 5 μ g of kidney, 5 μ g of small intestine, 2.5 μ g of adipose tissue, and 10 μ g of testis. mCES1/Sf9 was used as a positive control.

Total RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Three 8-week-old C57BL/6 male mice (Japan SLC) were sacrificed, and the removed tissue samples of equal weight were pooled. Total RNA was extracted from each tissue (brain, thymus, lung, heart, liver,

kidney, small intestine, epididymal adipose tissue, and testis) using TRIzol reagent (Invitrogen). To prevent contamination with genomic DNA, the extracts were treated with DNase I (Takara Shuzo, Shiga, Japan). Subsequently, first-strand cDNA was synthesized from 2 μ g of each RNA by Ready-To-Go RT-PCR Beads with an oligo(dT) primer (Amersham Biosciences Inc.). For certification of synthesis of equal amounts of cDNA, PCR was performed (94°C for 15 s, 49°C for 20 s, 68°C for 40 s, and 35 cycles) using a set of primers for detection of *mouse glyceraldehyde-phosphate dehydrogenase (GAPDH)* gene expression: sense, 5'-TGCACCACCAACTGCTTA-3'; and anti-sense, 5'-GGATGCAGGGATGATGTTC-3'. Another set of primers (sense, 5'-GGCATCAACAAGCAAGAGTTTGGC-3'; and anti-sense, 5'-CTTTTTGGTGAGGTGATCTGTCCC-3') was used for PCR (94°C for 15 s, 54°C for 20 s, 68°C for 35 s, and 32 cycles) to detect *mCES1* gene expression. The pFAST BAC1 vector containing mCES1 cDNA was used as a template for a positive control. The PCR product was purified by a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) and was confirmed to be a fragment of mCES1 cDNA by DNA sequencing. The DNA sequence was determined as described above.

Other Methods. SDS-PAGE was performed with 10% acrylamide gel, and Western blotting was performed by using a Vectrastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's directions. Mouse CES isozymes, CES ML1 and CES ML2, were purified by the same method as that used for the purification of mouse CES1 (M. Hosokawa, T. Furihata, N. Koyano, T. Satoh, and K. Chiba, manuscript in preparation). Chemicals used in this study were of reagent grade and obtained from commercial sources.

Results

Induction of Hydrolase Activity in Mouse Liver Microsomes by DEHP. Exposure of C57BL/6 mice to DEHP in their diet resulted in significant induction of hydrolase activity in mouse liver microsomes (Fig. 1). Compared with control Ms, DEHP Ms showed 3.0-fold ($P < 0.05$) and 2.8-fold ($P < 0.05$) increases in the level of PNPA hydrolase activity and temocapril hydrolase activity, respectively.

Purification of Mouse CES1 from DEHP-Treated Mouse Liver Microsomes and N-Terminal Amino Acid Sequences. Our purification procedures gave a purified CES isozyme indicated by a single band in SDS-PAGE (Fig. 2), and we tentatively named it mouse CES1. The isoelectronic point of mouse CES 1 was 5.8, which was estimated by chromatofocusing. Mouse CES1 was present as a trimer in Sephadex G-150 column chromatography (data not shown), and the molecular weight of a subunit of mouse CES1 was approximately 60,000, estimated by a plot of relative mobility versus log of the molecular weight of the standards. Binding of mouse CES1 to Con A-Sepharose indicated that mouse CES1 was a glycoprotein. The amino acid sequence of mouse CES1 was determined for the first 20 N-terminal amino acids, as shown in the box in Fig. 4.

Substrate Specificity of Mouse CES1. Specific activities of mouse CES1 toward some xenobiotics were examined. The level of activity of mouse CES1 toward PNPEsters was found to be dependent on the carbon chain length. When the carbon chain length of PNPEsters was three (PNPP) or four (PNPB), the activities toward PNPP and PNPB were almost the same (179 and 157 μ mol/mg protein/min, respectively), whereas both of them were much higher than that to PNPA (80.9 μ mol/mg protein/min), the carbon chain length of which was two. Mouse CES1 showed butanilicaine hydrolase activity (17.2 μ mol/mg protein/min) in addition to temocapril hydrolase activity (197 nmol/mg protein/min). Kinetic parameters for these substrates could not be determined due to the limited amount of purified enzyme available.

Specific Immunocrossreactivity of anti-CES D1 Antibodies with Mouse CES1. To obtain antibodies specific to mouse CES1, we performed antibody screening by Western blotting (Fig. 3). Two purified mouse CES isozymes (termed CES ML1 and CES ML2)

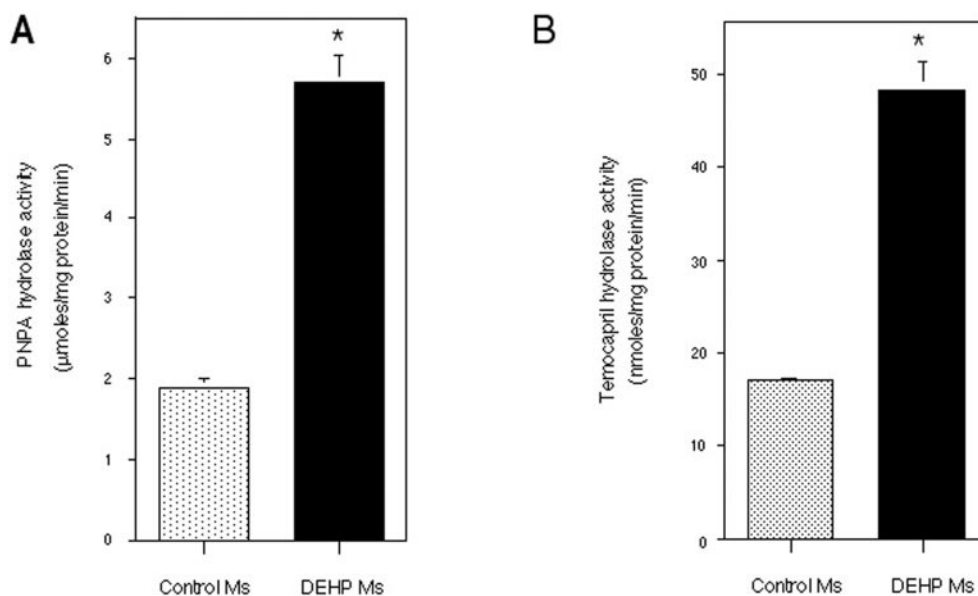


FIG. 1. Induction of carboxylesterase activity in DEHP-treated mouse liver microsomes. A, PNPA hydrolase activity; B, temocapril hydrolase activity. The sample number of control Ms or DEHP Ms was three. Each value is represented as the mean of three independent assays (\pm S.D.). * indicates statistically significant difference compared with the control Ms ($P < 0.005$).

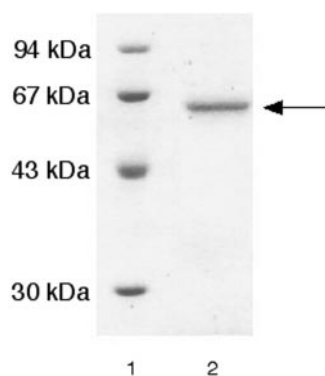


FIG. 2. Purification of mouse CES1 from DEHP-treated mouse liver microsomes. SDS-PAGE of purified preparations resulted in a single band. Lane 1, protein marker (94 kDa, phosphorylase *b*; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase). Lane 2, purified preparations (250 ng). An arrow indicates the purified mouse CES1.

were used in addition to mouse CES1 and mCES2 (Furihata et al., 2003) for the screening. Among CES antibodies, anti-CES D1 antibodies showed specific immunocrossreactivity with mouse CES1 (Fig. 3A, upper column), whereas anti-CES P1 antibodies recognized all four CES isozymes (Fig. 3A, lower column), although the recognition of mCES2 was weak. Anti-CES RL1 antibodies could recognize both mouse CES1 and CES ML1 but not CES ML2 or mCES2 (data not shown). Anti-CES D1 antibodies could detect mouse CES1 in both control Ms and DEHP Ms, and the intensity of the band in DEHP Ms was stronger than that in control Ms (Fig. 3B). Anti-CES D1 antibodies also detected an unknown protein with a slower migration point.

Effects of Specific IgG on Temocapril Hydrolase Activity in Mouse Liver Microsomes. After the IgG fraction had been purified from whole antibodies, we observed the effects of specific IgGs, anti-CES D1 IgG or anti-CES RL1 IgG, on temocapril hydrolase activity. The levels of activity in control Ms and DEHP Ms obtained from assays using control IgG were adjusted to 100% in each group. Inhibition by anti-CES D1 IgG caused 66.6% ($P < 0.05$) and 46.9%

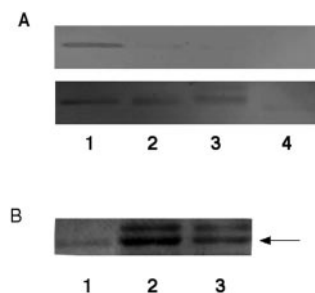


FIG. 3. Specific immunocrossreactivity of anti-CES D1 antibodies with mouse CES1. Western blotting was performed to identify antibodies that specifically recognize mouse CES1. The arrow indicates the band for mouse CES1. A, anti-CES D1 and anti-CES P1 antibodies were used in the upper and lower panels, respectively. 1, purified mouse CES1 (0.5 μ g); 2, purified mouse CES ML1 (0.5 μ g); 3, purified mouse CES ML2 (0.5 μ g); 4, purified mCES2 (0.5 μ g). B, anti-CES D1 antibodies were used. 1, purified mouse CES1 (0.5 μ g); 2, solubilized fraction of DEHP Ms (0.3 μ g); 3, control Ms (0.3 μ g).

($P < 0.05$) reductions in the level of temocapril hydrolase activity in control Ms and DEHP Ms, respectively. On the other hand, inhibition by anti-CES RL1 IgG resulted in 93.7% ($P < 0.05$) and 94.6% ($P < 0.05$) reductions in the activity level in control Ms and DEHP Ms, respectively.

Antibody Screening of cDNA Library. As a result of cDNA library screening by anti-CES RL1 antibodies, 27 positive clones were isolated from 9.6×10^4 plaques tested. Among them, a 1946-bp cDNA clone was found to contain an open reading frame encoding an amino acid polypeptide followed by a termination codon (TAG) and then by 206 nucleotides of a 3'-noncoding sequence including a poly(A) tail (data not shown). This cDNA clone was named mCES1. The deduced amino acid sequence of mCES1 is shown in Fig. 4. It contained a structurally important Cys residue (Cys99), catalytic triad (Ser204, Glu336, and His449), oxyanion hole loop (Gly124 and Gly125), and HXEL-motif (His562, Val563, Glu564, and Leu565), which are specific sequences conserved in the mammalian CES family (Sato and Hosokawa, 1998). The deduced amino acid sequence of mCES1 also contained two *N*-linked glycosylation sites (Asn62, Thr63, and Thr64; and Asn472, Leu473, and Ser474).

mCES1	1	MGLYPLIWL SLAACTAW-GYFSSPPVNTVTKGKYLKGYV	99
CESD1	1	MWLFDLVLTSLATSMAW-GYFSLP PVTVTGKVLGKYV	99
M-LK	1	MFLSTLFLVSLATKIC-GNPSPPVVDTHGKVLGKHYVTEGFSQPVAVFLGIFFAKPLGSLRFAPPQPAEPWFSVKNTTSYPPMCSDAARGQAVND	99
hCE1	1	WLRAFILATLSASAAWAGHPSSPPVVDVTHGKVLGKHYVTEGFSQPVAVFLGIFFAKPLGSLRFAPPQPAEPWFSVKNTTSYPPMCTQDAAGQQLLSE	100
Rat pl6.1	1	MRLYPLVWFLPAACTAW-GYFSSPPVNTVTKGKYLKGYV	99
mCES1	100	LFTNRKENIPLQF SEDCLYLNIYTPADLTKNRSLPVMVWIHGGGLV VGGASTYDGLALSAHENVVVTTIQYRLGIWGF STGDEHSRGNWGHLDQVAALR	199
CESD1	100	LFTNRKDNIPKLF SEDCLYLNIYTPADLTKNRSLPVMVWIHGGGLV VGGASTYDGLALSAHENVVVTTIQYRLGIWGF STGDEHSRGNWGHLDQVAALR	199
M-LK	100	LFTNRKEKIHLEF SEDCLYLNIYTPADLTKNRSRLPVMVWIHGGGLV VGGASTYDGLALSAHENVVVTTIQYRLGIWGF STGDEHSRGNWGHLDQVAALR	199
hCE1	101	LFTNRKENIPLKLF SEDCLYLNIYTPADLTKNRSLPVMVWIHGGGLV VGGASTYDGLALSAHENVVVTTIQYRLGIWGF STGDEHSRGNWGHLDQVAALR	200
Rat pl6.1	100	LFTNRKENIPLQF SEDCLYLNIYTPADLTKNRSLPVMVWIHGGGLV VGGASTYDGLALSAHENVVVTTIQYRLGIWGF STGDEHSRGNWGHLDQVAALR	199
mCES1	200	WVQDNIAFNGGNPGSVTIFGESAGGSV SVLVL SFLAKNLFHRAISESGVSLTAALITT-DVKFIAGLVATLSGCKTTTSAVMWHCLRQKTEDELLETSL	298
CESD1	200	WVQDNIAFNGGNPGSVTIFGESAGGSV SVLVL SFLAKNLFHRAISESGVSLTAALITT-DVKFIAGLVATLSGCKTTTSAVMWHCLRQKTEDELLETSL	298
M-LK	200	WVQDNIAFNGGNPGSVTIFGESAGGSV SVLVL SFLAKNLFHRAISESGVSLTAALITT-DVKFIAGLVATLSGCKTTTSAVMWHCLRQKTEDELLETSL	298
hCE1	201	WVQDNIAFNGGNPGSVTIFGESAGGSV SVLVL SFLAKNLFHRAISESGVSLTAALITT-DVKFIAGLVATLSGCKTTTSAVMWHCLRQKTEDELLETSL	300
Rat pl6.1	200	WVQDNIAFNGGNPGSVTIFGESAGGSV SVLVL SFLAKNLFHRAISESGVSLTAALITT-DVKFIAGLVATLSGCKTTTSAVMWHCLRQKTEDELLETSL	298
mCES1	299	KLNLFKLDDLGNPKESYFPLPTVIDGVVLPKAPPEILAEKSFSTVPIYVGINQKQFEGWIPTLMGYP LAEGKLD-QKTANSLWKSYP TLKISERMIPIV	397
CESD1	299	KLNLFKLDDLGNPKESYFPLPTVIDGVVLPKAPPEILAEKSFSTVPIYVGINQKQFEGWIPTLMGYP LAEGKLD-QKTANSLWKSYP TLKISERMIPIV	397
M-LK	299	KLNLFKLDDLGNPKESYFPLPTVIDGVVLPKAPPEILAEKSFSTVPIYVGINQKQFEGWIPTLMGYP LAEGKLD-QKTANSLWKSYP TLKISERMIPIV	398
hCE1	301	KMKFLSLDLQDPPRESQPLGTVIDGMLLKTPEELQAERNFHTVPIYVGINQKQFEGWILPMLQMSYPLSEGQLDQKTAHSLWKSYP LVCIAKELIPEA	400
Rat pl6.1	299	KLNLFKLDDLGNPKESYFPLPTVIDGVVLPKAPPEILAEKSFSTVPIYVGINQKQFEGWIPTLMGYP LAEGKLD-QKTANSLWKSYP TLKISERMIPIV	397
mCES1	398	AEKYLGTTDDLT KKKDLFQDLMDADV FGVPSVIVSRSHRDAGASTYNYEFYRPF SFV SAMRPAKVI GDHGD E L F SVFGAPFLKDGASEEETNL SKITVMKF	497
CESD1	398	SEKYLGGTDDP VKKALFLDLDLGDV FGVPSVIVSRSHRDAGASTYNYEFYRPF SFV SAMRPAKVI GDHGD E L F SVFGAPFLKDGASEEETNL SKITVMKF	497
M-LK	399	SEKYLGGTDDP VKKALFLDLDLGDV FGVPSVIVSRSHRDAGASTYNYEFYRPF SFV SAMRPAKVI GDHGD E L F SVFGAPFLKDGASEEETNL SKITVMKF	497
hCE1	401	TEKYLGGTDDP VKKALFLDLDLGDV FGVPSVIVSRSHRDAGASTYNYEFYRPF SFV SAMRPAKVI GDHGD E L F SVFGAPFLKDGASEEETNL SKITVMKF	500
Rat pl6.1	398	AEKYFGTDDP AKRDLFQDLVADV FGVPSVIVSRSHRDAGASTYNYEFYRPF SFV SAMRPAKVI GDHGD E L F SVFGAPFLKDGASEEETNL SKITVMKF	497
mCES1	498	WANFARNGNPNGGGLPHWPEYDQKEGYLKGASTQAQRLLKQKEV SFWAE LRAKE SAQR SHREHVEL	565
CESD1	498	WANFARSGNPNGGGLPHWPEYDQKEGYLKGASTQAQRLLKQKEV SFWAE LRAKE SAQR SHREHVEL	565
M-LK	498	WANFARNGNPNGGGLPHWPEYDQKEGYLKGASTQAQRLLKQKEV SFWAE LRAKE SAQR SHREHVEL	561
hCE1	501	WANFARNGNPNGGGLPHWPEYDQKEGYLKGASTQAQRLLKQKEV SFWAE LRAKE SAQR SHREHVEL	568
Rat pl6.1	498	WANFARNGNPNGGGLPHWPEYDQKEGYLKGASTQAQRLLKQKEV SFWAE LRAKE SAQR SHREHVEL	565

Fig. 4. Comparison of the deduced amino acid sequence of mCES1 with those of other CES1 isozymes. Shadows indicate differences in amino acid residues. The box indicates the same sequence as that of purified mouse CES1 identified. Stars indicate residues composing a catalytic triad. Square indicates structurally important Cys residues. Circles indicate two Gly residues important for oxianion hole loop formation. Asterisks indicate endoplasmic retention signal motifs. Dashes indicate *N*-linked glycosylation sites.

Baculovirus-Mediated Expression of mCES1 in Sf9 Cells and Carbon Chain Length Specificity of the Recombinant mCES1 Protein. Western blotting showed that the recombinant mCES1 protein was recognized by anti-CES D1 antibodies and that the molecular weight of a subunit was the same as that of purified mouse CES1 (Fig. 5A). Esterase activity staining after nondenaturing PAGE also showed that the recombinant CES1 protein possessed α -naphthylacetate hydrolase activity, and the migration rate of the protein was the same as that of purified mouse CES1 (Fig. 5B). On the other hand, a specific protein was not detected in mock/Sf9 either by Western blotting or esterase activity staining after nondenaturing PAGE. In esterase activity staining after nondenaturing PAGE, the accessory band found at a faster migration point indicated by an asterisk was thought to be a monomeric form of mCES1 (Robbi and Beaufay, 1991).

To analyze the effect of carbon chain length of the substrate on hydrolase activity of the recombinant mCES1, we used PNPesters that contained various carbon chain lengths (from 2 to 12) as substrates (Fig. 5C). The level of specific activity in mCES1/Sf9 was the highest at the length of four. mCES1/Sf9 also showed high levels of activity at lengths from two to five. However, the level of activity decreased as the carbon chain of PNPesters became longer, and when the substrate was *p*-nitrophenyllaurate, the activity level in mCES1/Sf9 was almost the same as that in mock/Sf9. According to the results of Western blotting and esterase activity staining after nondenaturing PAGE, mock/Sf9 showed a significantly low level of activity toward PNPesters.

Substrate Specificity of Recombinant mCES1 Protein. Kinetic parameters of PNPA, temocapril, and butanilicaine hydrolase activity in mCES1/Sf9 were analyzed. Since the levels of activity toward PNPA, temocapril, and butanilicaine in mock/Sf9 were significantly low, if any, the background activity in mCES1/Sf9 was negligible. K_m value and V_{max} value of recombinant mCES1 to PNPA were $21.3 \pm$

$3.05 \mu\text{M}$ and $2.72 \pm 0.09 \mu\text{mol/mg protein/min}$, respectively. As purified mouse CES1 did, recombinant mCES1 showed capabilities to hydrolyze temocapril as well as butanilicaine. The K_m value and V_{max} value to temocapril were $389 \pm 62.4 \mu\text{M}$ and $9.64 \pm 1.28 \text{ nmol/mg protein/min}$, and those to butanilicaine were $518 \pm 19.0 \mu\text{M}$ and $430 \pm 17.1 \mu\text{mol/mg protein/min}$, respectively.

Tissue Expression Profiles of mCES1 mRNA and Protein. RT-PCR and esterase activity staining after nondenaturing PAGE were performed to obtain tissue expression profiles of mCES1 mRNA and protein, respectively. The results of PCR using mouse *GAPDH* gene-specific primers showed that genomic DNA did not mingle with mouse tissue cDNAs (Fig. 6A) and that the cDNAs were synthesized in equal amounts (Fig. 6B). PCR using *mCES1* gene-specific primers revealed that mCES1 mRNA expression was present in the thymus, lung, heart, liver, kidney, small intestine, adipose tissue, and testis (Fig. 6C). Expression of mCES1 mRNA was not detected in the brain. Nucleotide sequences of all of the PCR products showed 100% homology with that of mCES1 cDNA. In accordance with the results of RT-PCR, esterase activity staining after nondenaturing PAGE showed that activity of mCES1 protein was present in the thymus, lung, heart, liver, kidney, adipose tissue, and testis (Fig. 6D). There was no band observed in the lane of the brain and small intestine at the same position as that of mCES1 protein. As in the case of esterase activity staining for which results are shown in Fig. 5B, we found an accessory band at a faster migration point, which is thought to be a monomeric form of mCES1 (Robbi and Beaufay, 1991), indicated by an asterisk.

Discussion

The enzyme responsible for temocapril hydrolysis in mouse liver microsomes has not been identified. In our preliminary work, a CES-specific inhibitor (Brandt et al., 1980), bis (*p*-nitrophenyl) phos-

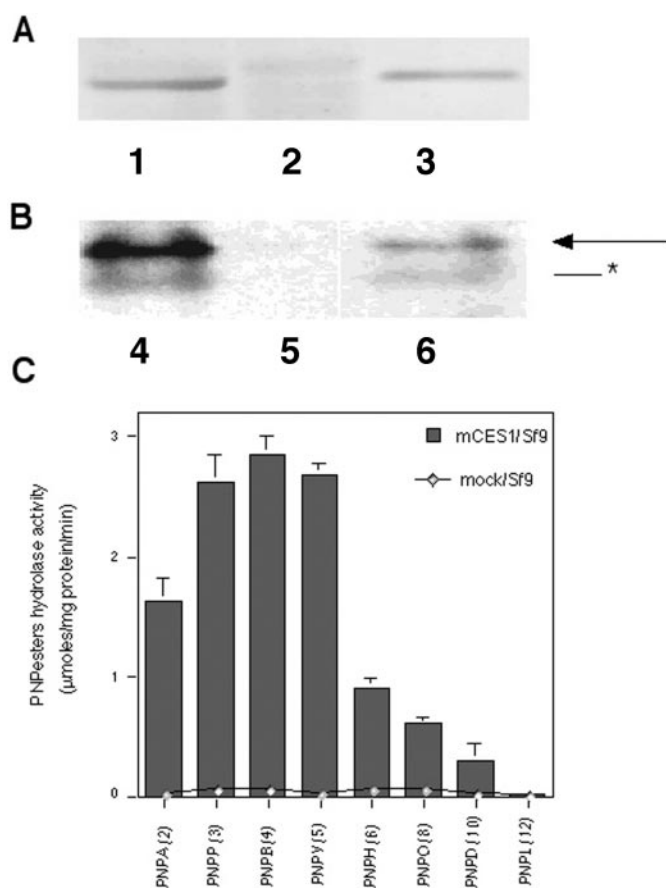


FIG. 5. Validation of recombinant mCES1 expression in Sf9 cells and chain length specificity for PNPester hydrolase activity. Western blotting (in panel A) and esterase activity staining after nondenaturing PAGE (in panel B) were performed to demonstrate the expression of mCES1 in Sf9 cells. Lanes 1 and 4, mCES1/Sf9 (5 μ g and 7.2 μ g, respectively); lanes 2 and 5, mock/Sf9 (5 μ g and 26 μ g, respectively); lanes 3 and 6, purified mouse CES1 (0.7 μ g and 70 ng, respectively). Panel C shows the hydrolase activities of mCES1/Sf9 (bar chart) and mock/Sf9 (polygonal line) for PNPesters that contain carbon chains of various lengths, indicated in parentheses after the substrate name.

phate, strongly inhibited temocapril hydrolase activity in mouse liver microsomes (by more than 90%) (data not shown). It has also been reported that temocapril can be a good substrate for hCE-1 (Takai et al., 1997; Mori et al., 1999). The temocapril hydrolase activity level of mCES2 expressed in Sf9 cells was under the detectable level (data not shown), although we have recently reported that one of the CES isozymes induced by DEHP treatment is mCES2 (Furihata et al., 2003). Thus, it is thought that the large increase in the level of temocapril hydrolase activity in liver microsomes of mice treated with DEHP was due to the induction of a mouse CES1 isozyme(s), and we identified the mouse CES1 isozyme by purification, cDNA cloning, and functional expression in Sf9 cells as described below. The identification of the mouse CES1 isozyme is important for the study of drug metabolism since the mouse CES1 isozyme exhibits striking similarities to the human CES1 isozyme.

As predicted, significant contribution of mouse CES1 to temocapril hydrolase activity in both control Ms and DEHP Ms was demonstrated by inhibition assays using specific antibodies (Fig. 7). The assays also demonstrated that 1) another mouse CES isozyme(s) is involved in temocapril hydrolysis in liver microsomes, 2) unidentified CES isozyme(s) was also induced by DEHP treatment, and 3) almost all of the temocapril hydrolysis activity in mouse liver microsomes is due to CES isozymes, including mouse CES1. Collectively, these

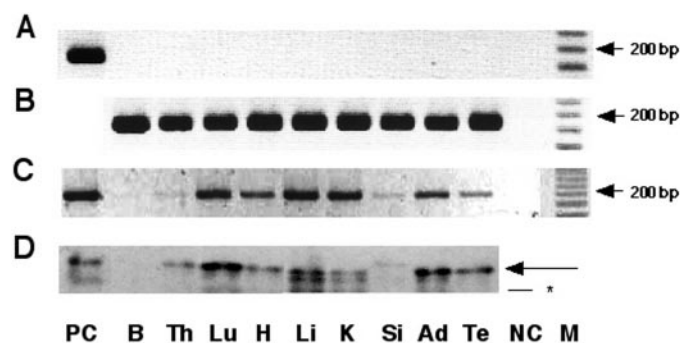


FIG. 6. Tissue expression profiles of mCES1 mRNA and protein. Panels A to C show the results of RT-PCR, and panel D shows the results of esterase activity staining after nondenaturing PAGE. PC, positive control; B, brain; Th, thymus; Lu, lung; H, heart; Li, liver; K, kidney; Si, small intestine; Ad, adipose tissue; Te, testis; NC, nontemplate control; M, DNA size marker. In panel A, mouse *GAPDH* gene-specific primers were used and mouse tissue RNA was used as a template. Mouse liver cDNA prepared in a previous study (Furihata et al., 2003) was used as a positive control. In panel B, mouse *GAPDH* mRNA expression was detected by using mouse tissue cDNAs as templates. In panel C, mCES1 mRNA expression was detected by using mouse tissue cDNAs as templates. mCES1 cDNA in the pFAST-BAC1 vector was used as a positive control. Panel D shows mCES1 protein activity. Purified mouse CES1 (70 ng) was used as a positive control. Various amounts of mouse tissue homogenates were used: 20 μ g of brain, 15 μ g of thymus, 7 μ g of lung, 10 μ g of heart, 1 μ g of liver, 5 μ g of kidney, 5 μ g of small intestine, 2.5 μ g of adipose tissue, and 10 μ g of testis homogenates. An arrow indicates the position of mCES1, and an asterisk indicates the accessory band.

results indicated that mouse CESs play an important role in ester-prodrug metabolism. Other mouse CES isozymes involved in temocapril hydrolysis remain unidentified. However, anti-CES RL1 antibodies could recognize CES ML1 as well as mouse CES1 (data not shown); thus, one possibility is that CES ML1 also contributes to temocapril hydrolysis in mouse liver microsomes. Further experiments are needed to determine other CES isozymes involved in temocapril hydrolysis and to determine whether they are induced by DEHP.

Given that mouse CES1 was involved in DEHP-induced temocapril hydrolase activity in mouse liver microsomes, the next step was characterization of purified mouse CES1. Biochemical characteristics of purified mouse CES1 are very similar to those of mammalian CESs (Sato and Hosokawa, 1998). We should emphasize that purified mouse CES1 was present as a trimer, which is a specific characteristic of CES1A subfamily members. This subfamily includes hCE-1, CES D1 (Hosokawa et al., 2001), and CES RH1 (also called p16.1 or ES-10) (Hosokawa et al., 1987; Robbi et al., 1990) (Fig. 8). All of these members have been shown to form trimers, whereas other family members behave as monomers (Sato and Hosokawa, 1998). The N-terminal amino acid sequence and immunological properties of purified mouse CES1 provided further experimental data showing that mouse CES1 is similar to other CES1A subfamily isozymes. The N-terminal amino acid sequence of mouse CES1 showed 100% homology to that of CES RH1/p16.1, 85% homology to that of CES D1, and 80% homology to that of hCE-1 (Fig. 4). The finding that antibodies against CES D1 could specifically recognize mouse CES1 suggested that structural properties of these CES isozymes are similar. Taken together, the results suggest that mouse CES1 belongs to the CES1A subfamily.

After protein characterization, the next issue was identification of the nucleotide sequence encoding mouse CES1. After we had submitted the nucleotide sequence of the obtained cDNA clone, mCES1, to DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank nucleotide sequence databases (AB023631), another group reported cDNA coding mouse triacylglycerol hydrolase

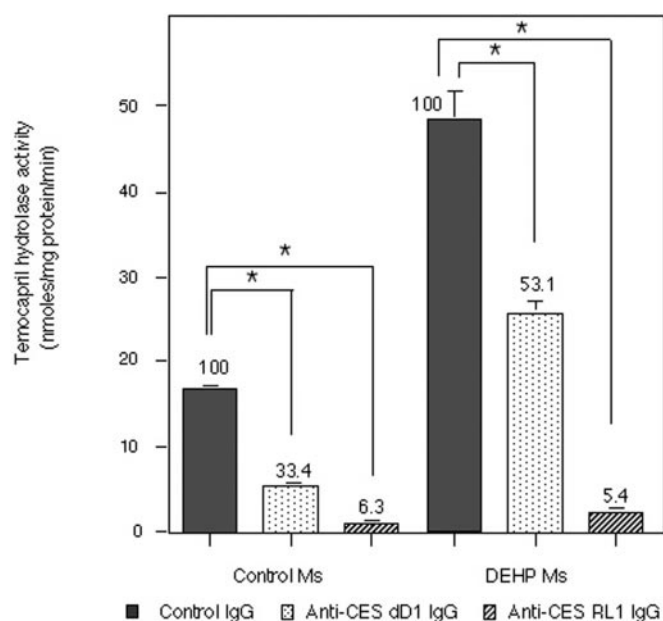


FIG. 7. Effects of anti-CES IgGs on temocapril hydrolase activity in control Ms or DEHP Ms. Control IgG (0.75 mg), anti-CES D1 IgG (0.75 mg), and anti-CES RL1 IgG (0.75 mg) were used (solid bar, dotted bar, and hatched bar, respectively). The sample number of control Ms or DEHP Ms was three. Each value is the mean of three independent assays (\pm S.D.). The number above each bar is relative percentage of activity when that of control IgG in each group (control Ms or DEHP Ms) is set to 100%. * indicates statistically significant difference compared with the results of control IgG in each group ($P < 0.005$).

(mTGH) (AF378751) (Dolinsky et al., 2001). Except for one nucleotide in the region coding signal peptide, the nucleotide sequence of mTGH showed perfect homology to that of mCES1 (data not shown). In addition, other biochemical properties of mTGH (Dolinsky et al., 2001) are very similar to those of mCES1 characterized in this study; thus, mCES1 and mTGH are considered to be the same enzymes with different names. This accordance is not surprising since recent studies have suggested that CESs are involved in fatty acid ester metabolism (Schindler et al., 1998; Hosokawa et al., 2001; Ito et al., 2002; Furihata et al., 2003) and should be useful information for elucidating physiological functions of CESs that remain unknown.

Overall homologies of the deduced amino acid sequence of mCES1 to CES1A subfamily isozymes were higher (92.7% to CES RH1/pl6.1, 79.6% to CES D1, and 77.6% to hCE-1) than those to CES1B subfamily isozymes (71.3% to hydrolase C, 70.3% to mouse liver and kidney carboxylesterase, and 69.4% to CES RL1), and mCES1 showed poor homology to CES2 isozymes (less than 50%; data not shown). Therefore, mCES1 is thought to encode a mouse CES1A subfamily isozyme.

The deduced amino acid sequence of mCES1 contained the same sequence as the N-terminal amino acid sequence of purified mouse CES1, indicating that mCES1 encodes mouse CES1. The results of a series of experiments using recombinant mCES1 supported this speculation. Recombinant mCES1 protein exhibited biochemical characteristics, such as molecular weight or hydrolytic activity toward temocapril and butanilcaine, identical to those of purified mouse CES1. Based on all of our data, it is concluded that mCES1 encodes mouse CES1, belonging to the CES1A subfamily as shown in Fig. 8.

Considering our finding that mCES1 was greatly involved in temocapril metabolism, tissue expression profiles of mCES1 should be clarified. RT-PCR was not a perfect quantitative method for mRNA expression. Nevertheless, the amplification levels of the specific band were apparently different between some tissue samples. Expression of

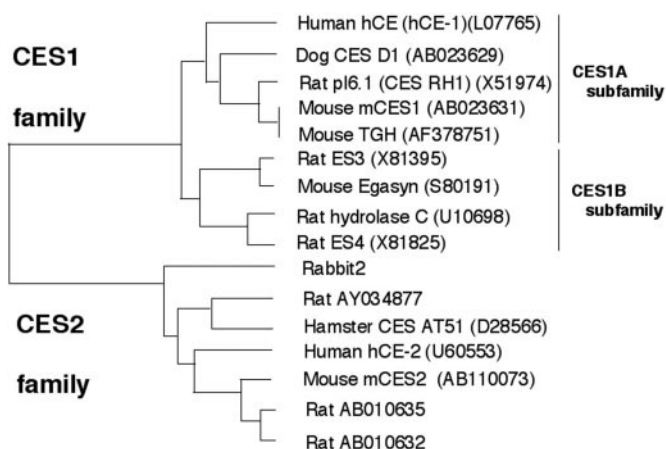


FIG. 8. Phylogenetic tree of the CES family. The phylogenetic tree was created using a simple unweighted pair-group method of analysis dendrogram. The trivial name, species, and gene bank accession number for their cDNA are shown.

mCES1 in the lung and kidney is noteworthy since these tissues are important for extrahepatic drug metabolism. It is notable that overall expression profiles of mCES1 are very similar to those of hCE-1, which is expressed in the brain, heart, lung, liver, and testis (Satoh et al., 2002; Xie et al., 2002), although there are a few discrepancies in their expression profiles, such as the case in the brain. Together with our other results, it is considered that mCES1 and hCE-1 are very similar enzymes. This may reflect functional similarities of these two enzymes in drug metabolism and fatty acid ester metabolism.

Given that mCES1 is an important CES1 isozyme involved in drug metabolism like hCE-1, we should refer to mechanisms by which DEHP induced mCES1, since the induction of drug metabolism enzymes has considerable significance in pharmacokinetics and pharmacological actions of drugs metabolized by the enzymes. DEHP, a peroxisome proliferator (PP), has been shown to cause hepatomegaly and peroxisome proliferation, and eventually, hepatocarcinogenesis, in rodents (Huber et al., 1996). Recent studies have shown that many effects of DEHP in the rodent liver were mediated by peroxisome proliferator-activated receptor α (PPAR α) (Ward et al., 1998). Thus, it can be speculated that the induction of mCES1 by DEHP is mediated by PPAR α , and it has been reported that the *mCES1* promoter has a PPAR response element-like motif (Douglas et al., 2001). However, the participation of PPAR α in the regulation of *mCES1* gene expression is controversial. Hosokawa and colleagues (Hosokawa et al., 1988; Hosokawa and Satoh, 1993) reported that oral administration of clofibrate or perfluorinated fatty acids to rats for 3 days or 5 days, respectively, resulted in an increase in the level of CES RH1/pl6.1 expression. On the other hand, Dolinsky et al. (2003) have recently reported that 2 weeks of clofibrate feeding did not significantly affect mCES1 expression in wild-type or PPAR α -null mice. Poole et al. (2001) showed that the changes in expression level of CES RH1/pl6.1 caused by PP treatment were time- and compound-dependent. The alteration of mCES1 expression level by PP in the liver may result from an adaptive secondary effect, and further studies are needed to understand the molecular mechanisms of PP by which mCES1 is induced.

In this study, we identified a mouse CES1 isozyme, mCES1, that was induced by DEHP. Purification, cDNA cloning, and baculovirus-mediated expression of mCES1 revealed that mCES1 plays an important role in temocapril metabolism and that it belongs to the CES1A subfamily. Collectively, our results showed that mCES1 is very similar to hCE-1. Therefore, mCES1 is thought to be one of the critical determinants for pharmacokinetics and pharmacological ac-

tions of ester prodrugs as well as ester drugs. This work provides useful information for study of metabolism and disposition of ester-prodrugs as well as ester-drugs. Elucidation of the substrate specificity of *mCES1* and the mechanisms by which the *mCES1* gene is regulated is our next challenge.

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