Molecular Cloning and Characterization of Two Mouse Peroxisome Proliferator-activated Receptor α (PPARα)-regulated Peroxisomal Acyl-CoA Thioesterases*

Maria A. K. Westin, Stefan E. H. Alexson, and Mary C. Hunt†

From the Department of Laboratory Medicine, Karolinska Institutet, Division of Clinical Chemistry, C1-74, Karolinska University Hospital at Huddinge, SE-141 86 Stockholm, Sweden

Peroxisomes are organelles that function in the β-oxidation of long- and very long-chain acyl-CoAs, bile acid-CoA intermediates, prostaglandins, leukotrienes, thromboxanes, dicarboxylic fatty acids, pristanic acid, and xenobiotic carboxylic acids. The very long- and long-chain acyl-CoAs are mainly chain-shortened and then transported to mitochondria for further metabolism. We have now identified and characterized two peroxisomal acyl-CoA thioesterases, named PTE-Ia and PTE-Ic, that hydrolyze acyl-CoAs to the free fatty acid and coenzyme A. PTE-Ia and PTE-Ic show 82% sequence identity at the amino acid level, and a putative peroxisomal type 1 targeting signal of -AKL was identified at the carboxyl-terminal end of both proteins. Localization experiments using green fluorescent fusion protein showed PTE-Ia and PTE-Ic to be localized in peroxisomes. Despite their high level of sequence identity, we showed that PTE-Ia is mainly active on long-chain acyl-CoAs, whereas PTE-Ic is mainly active on medium-chain acyl-CoAs. Lack of regulation of enzyme activity by free CoASH suggests that PTE-Ia and PTE-Ic may regulate intraperoxisomal levels of acyl-CoA, and they may have a function in the termination of β-oxidation of fatty acids of different chain lengths. Tissue expression studies revealed that PTE-Ia is highly expressed in kidney, whereas PTE-Ic is most highly expressed in spleen, brain, testis, and proximal and distal intestine. Both PTE-Ia and PTE-Ic were highly up-regulated in mouse liver by treatment with the peroxisome proliferator WY-14,643 and by fasting in a peroxisome proliferator-activated receptor α-dependent manner. These data show that PTE-Ia and PTE-Ic have different functions based on different substrate specificities and tissue expression.

Peroxisomes are organelles present in most eukaryotic cells and play a key role in the metabolism of a variety of lipids such as very long-chain fatty acids, dicarboxylic fatty acids, bile acids, prostaglandins, leukotrienes, thromboxanes, pristanic acid, and xenobiotic fatty acids and in the biosynthesis of ether-linked glycerolipids such as plasmalogens and structural ether lipids abundant in the central nervous system (for reviews, see Refs. 1 and 2). Peroxisomal β-oxidation of very-long- and long-chain CoA esters results in chain shortening of fatty acids, which may then be transported to the mitochondria as carnitine esters for further oxidation. β-Oxidation of other types of lipids such as prostanooids leads to chain shortening in the peroxisome for excretion as free carboxylic acids in the urine (3). Almost 30 years ago a group of compounds was identified that were shown to cause peroxisome proliferation in rodents; hence they were named peroxisome proliferators. Peroxisome proliferators are structurally diverse groups of compounds including plasticizers, hypolipidemic drugs (for example, clofibrate), and WY-14,643. Peroxisome proliferators induce the expression of a number of genes involved in degradation of fatty acids and cause peroxisome proliferation, hepatomegaly, and hepatocarcinogenesis in rodent liver (4). These effects are mediated via the peroxisome proliferator-activated receptor alpha (PPARα), which was shown to be a nuclear receptor in control of lipid metabolism (5, 6). PPARα induces the expression of many genes involved in peroxisomal and mitochondrial β-oxidation and α-oxidation of fatty acids, and targeted disruption of this receptor in mouse has established its key role as a mediator of lipid metabolism (6). Free fatty acids were identified as physiological, endogenous ligands for the PPARα (7–10), which probably mediates the effects of PPARα during fasting (11–14).

One group of enzymes previously identified as responsive to treatment by peroxisome proliferators was the acyl-CoA thioesterases (for review, see Ref. 15). These enzymes are found in various compartments in the cell, including cytosol, mitochondria, microsomes, and peroxisomes. Acyl-CoA thioesterases catalyze the hydrolysis of acyl-CoAs to the free fatty acid and coenzyme A, thereby regulating intracellular content of various acyl-CoAs, free fatty acids, and free CoASH. The acyl-CoA thioesterase activity in peroxisomes was characterized and activity was identified with acyl-CoA esters from C2 to C20-CoA, with highest activity detected for medium- and long-chain acyl-CoAs (16). Since then, several peroxisomal acyl-CoA thioesterases have been cloned and characterized. A family of acyl-CoA thioesterases was identified in mouse, named Type-I acyl-CoA thioesterase (17). In one mitochondrial (MTE-I), and one cytosolic (CTE-I), all of
Molecular Characterization of Peroxisomal Thioesterases

which are located in a cluster on mouse chromosome 12 (17). In human, four Type-I acyl-CoA thioesterases have been identified, with putative localizations in cytosol, mitochondria, and peroxisomes (17, 18). Another peroxisomal acyl-CoA thioesterase called PTE-2, which is unrelated to the Type-I gene family above, was identified as the major acyl-CoA thioesterase in mouse liver peroxisomes (19), with homologues in human, yeast (20–23). This acyl-CoA thioesterase that hydrolyzes almost all acyl-CoAs present in peroxisomes, including bile acid-CoA esters, short-, medium- and long-chain acyl-CoAs and intermediates from β-oxidation of pristanoyl-CoA. It is hypothesized that this enzyme is important in regulating intracellular levels of acyl-CoAs and CoASH during times of high β-oxidation and fatty acid overload.

In this study, we have cloned and characterized PTE-Ia and PTE-Ic, the latter of which is a newly identified member of the Type-I family of acyl-CoA thioesterases. Both these enzymes are peroxisomal, are highly homologous (>80% sequence identity at amino acid level), and hydrolyze long- and medium-chain acyl-CoAs, respectively. These enzymes may play a role in controlling acyl-CoA levels within the peroxisomes in different tissues.

EXPERIMENTAL PROCEDURES

Chemicals—All commercially available acyl-CoAs used in this study were from Sigma. Behenoyl-CoA, lignoceryl-CoA, ceroteryl-CoA, and dimethylmaleoyl-CoA were kind gifts from Dr. Ronald Wands. Animals and Treatments—All tissues used in this study were excised from adult male wild-type and PPARα-null mice on a pure Sv/129 genetic background (kindly provided by Dr. Frank Gonzalez and Dr. Jeffrey Peters). The mice were fed a standard chow diet (R38; Lactamin, Västena, Sweden) or alternatively fed a diet containing 0.1% WY-14,643 (Cibachioch-Novabiochem International) or fasted for 4 h. Animals were sacrificed by CO2 asphyxiation followed by cervical dislocation; tissues were excised and stored at −70 °C for preparation of total RNA.

cDNA Cloning and Expression—The putative open reading frames for PTE-Ia (17) and PTE-Ic were derived from genomic data bases and expressed sequence tags (ESTs) from the mouse EST database (www.ncbi.nlm.nih.gov), respectively, and were amplified using the following primers: for PTE-Ia, 5'-CATATGATGGTCTCTGTAATACTACC-3' and 5'-CATATGGCGGCTTACCC-3' (which amplifies a splice variant named PTE-Ia 5:1; see below), and for PTE-Ic using 5'-CATATGGACACTCTCTAACAAGAAATCAT-3' and 5'-CATATGGTGCACACAGTAAAGCCT-3' (Cybergene AB, Huddinge, Sweden) with the addition of an NdeI site (indicated in bold). The full-length open reading frames were amplified by reversed transcription-PCR (RT-PCR) using a template of WY-treated mouse liver total RNA. RT-PCR was performed using the One-Step RNA PCR kit (avian myeloblastosis virus) (Takara Biomedicals). Thermal cycling for PTE-Ia was performed for 40 cycles at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 2.5 min, followed by 72 °C for 10 min. For PTE-Ic, an annealing temperature of 55 °C, 4 min extension and 35 cycles was used. The open reading frames were cloned into the NdeI site of the pET-16b plasmid (Novagen Inc.) for cloning as fusion proteins with green fluorescent protein (GFP). The open reading frames of PTE-Ia (5:1) and PTE-Ic were amplified by RT-PCR, using the same primers used for cloning into the pET16b plasmid. Both PCR products were cloned into the pCDNA1/NT-GFP vector (Invitrogen) in-frame with the GFP at the amino-terminal end. Sequence analysis was performed on the cloned products. In addition, two carboxyl-termin- nal GFP constructs, one full-length construct, and one construct lacking the last 11 amino acids were cloned. These constructs were ampliﬁed using the following primers: 5'-AGGATGCGACCCATTACCA-AC-3' (5:1), 5'-AGGATGGCCACTACTGTGT-3' (5:2), and 5'-GTTTGGATGGATCTC-3' (common primer) and cloned into the pCDNA1/CT-GFP vector (Invitrogen) as described above.

Human skin ﬁbroblasts from a control subject and a Zellweger patient were grown as described previously (19). Both control and Zellweger cells were grown overnight in 60-mm dishes on glass coverslips and were transfected with 10 μg of PTE-Ia/GFP or PTE-Ic/GFP plasmid using the calcium phosphate method. Transfected cells were grown for 48 h, and cells were ﬁxed and prepared for immunofluorescence microscopy as described in Ref. 19. The cells were examined in a Leica DM RB fluorescence microscope using Hamamatsu C4742–95 Twa interface software.

Tissue Expression and Regulation by WY-14,643 and Fasting by RT-PCR—The tissue expression of PTE-Ia and PTE-Ic was examined in WY-treated livers from wild-type (+/+) and PPARα-null (−/−) mice. Regulation by fasting was examined in liver, kidney, heart, lung, adrenal, spleen, testis, muscle, brain, brown adipose tissue, white adipose tissue, proximal intestine, distal intestine, and gallbladder. The regulation by WY-14,643 was investigated in WY-treated livers from wild-type (+/+) and PPARα-null (−/−) mice. Regulation by fasting was examined in liver, kidney, and heart from wild-type and PPARα-null mice. PTE-Ia was ampliﬁed using the following primers: 5'-TGTTATGCACCCGCGTCACA-3' and 5'-GGGT-GCCACGAGCAGTCA-3' (representing a fragment of 505 bp from the 5′-end of the cDNA sequence, single primer) and cloned into the pET-16b plasmid, which will amplify both variants 5:1 and 5:2 of PTE-Ia, and PTE-Ic using 5'-AACATTATGCGCCACGCTCA-3' and 5'-CCACGCTATGCTGACT-3' (representing a 534-bp fragment from 900–1434 of the cDNA sequence). RT-PCR for PTE-Ia was performed for 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, followed by 72 °C for 2 min. For PTE-Ic, an annealing temperature of 56 °C, 3 min extension time of 2 min was used. A fragment of β-actin was ampliﬁed as a control using the following primers: 5'-ATGCGATCAGATGCGTGCGTC-3' and 5'-GGCGTACCTCTTACCGGGTGCTTATGCTC-3'. Thermal cycling was performed for 28 cycles with an annealing temperature of 65 °C and extension of 20 s.

For Blot Analysis of Tissue Expression and Regulation by WY-14,643 and Fasting—Tissue expression of PTE-Ia at protein level was examined in muscle, heart, lung, brown adipose tissue, brain, liver, and kidney from Sv/129 male mice. The regulation of expression by WY-14,643 and fasting was investigated in wild-type and PPARα-null mice.
Tissue pieces (about 0.1 g in 200 μl of buffer) were homogenized in 50 mM potassium phosphate buffer, pH 7.0. Fifty micrograms of protein was analyzed by Western blot as described (14), using an anti-rabbit PTE-Ia antibody (Sigma Genosys) generated against the first 15 amino acids of the protein, resulting in an antibody that specifically recognizes PTE-Ia antibody.

RESULTS

cDNA Cloning and Sequence Analysis—We previously identified and cloned a novel gene family of four Type-I acyl-CoA thioesterases with members identified in cytosol (CTE-I), mitochondria (MTE-I), and peroxisomes (PTE-Ia and PTE-Ib) (17). These genes are localized in a cluster on chromosome 12 in mouse. Recent EST data base searches have identified two further putative peroxisomal Type-I acyl-CoA thioesterases, namely PTE-Ic and PTE-Id, that are also located in the same cluster on mouse chromosome 12 (Fig. 1). Of the four putative peroxisomal genes identified in mouse, PTE-Ia and PTE-Ic show the highest homology to each other and were therefore cloned and characterized together in this study. The genomic organization for PTE-Ia and PTE-Ic was obtained from the mouse genomic data base (ENSEMBL, www.ensembl.org), as outlined in Fig. 2A. Both proteins are encoded by three exons, spaced by two introns. Analysis of EST sequences for PTE-Ia suggested the possible existence of an additional small exon (30–37 bp) further upstream in the 5’-end, which results in two different splice variants depicted as 5’:1 and 5’:2 in Fig. 2A. The existence of these two splice variants was further supported by sequence analysis because the exon-intron boundaries are consistent with the donor-acceptor splice rules, as shown in Fig. 2B. Furthermore, these two splice variants were amplified by RT-PCR from liver and kidney (see below).

Based on the genomic sequences, the corresponding open reading frames were amplified by RT-PCR and fully sequenced. The full-length cDNAs were assembled based on ESTs corresponding to the 5’- and 3’-untranslated regions and verified by analysis of mouse genomic sequences. Sequence analysis of the gene products for PTE-Ia and PTE-Ic shows a sequence identity of 82% at the amino acid level in their open reading frames (Fig. 3). The cDNAs isolated for PTE-Ia and PTE-Ic encode proteins of 432 and 421 amino acids, with calculated molecular masses of 47.4 kDa and 46.6 kDa, respectively. Interestingly, the PTE-Ia cDNA of the PTE-Ia 5’:1 splice variant contains an extra 11 amino acids in the amino-terminal end, whereas the 5’:2 splice variant splices out the first methionine, with the protein starting at the second methionine, similar to the cytosolic and other peroxisomal thioesterases (17). Both sequences end –AKL at their carboxyl-terminal, which is a variant of the peroxisomal consensus type 1 targeting signal (PTS1) of –SKL (25). A catalytic triad containing serine (S), histidine (H), and aspartic acid (D) residues, located at positions 232, 360, and 326 of PTE-Ia, has
skin fibroblasts were transfected with PTE-Ia under GFP and processed for immunofluorescence microscopy as outlined previously (Fig. 4A). However, in Zellweger fibroblasts, transfection of PTE-Ia resulted in a diffuse GFP expression showing that PTE-Ia remained in the cytosol (Fig. 4B). PTE-Ic also showed a punctate pattern of expression in control fibroblasts, although some cytosolic staining was visible in this case (Fig. 4C). Again, in Zellweger fibroblasts, transfection of PTE-Ic resulted in a diffuse GFP expression (Fig. 4D). Because of the 11-amino acid extension in the amino-terminal of PTE-Ia (5':1), we also examined the possibility of a mitochondrial targeting of PTE-Ia. This was examined by expressing both the long (5':1) and the short (5':2) amino-terminal variants of PTE-Ia as carboxyl-terminal GFP fusion proteins in fibroblasts. However, the GFP experiments for these variants showed only cytosolic labeling with no mitochondrial localization (data not shown). Recombinant Expression and Characterization—The cloning of the open reading frames encoding PTE-Ia (5':1) and PTE-Ic into the NdeI site in the pET16b vector results in expression of the proteins as His-tagged fusion proteins, which allows for purification using affinity chromatography. Following purification on a Hi-Trap™ column, the purified proteins were detected as single bands of about 47 kDa on SDS-PAGE stained with Coomassie Brilliant Blue (data not shown). Following initial enzyme activity characterization of the recombinant protein, it was evident that PTE-Ia activity, but not PTE-Ic activity, was inhibited with substrates ranging from C14-C20 at concentrations higher than 5–10 μM. However, addition of bovine serum albumin to the reaction at an albumin/acyl-CoA ratio of 1:4.5 prevented inhibition (data not shown). Recombinant PTE-Ia and PTE-Ic were analyzed for acyl-CoA thioesterase activity, which was determined at several concentrations for a variety of acyl-CoA substrates, including saturated and unsaturated straight-chain acyl-CoAs, branched-chain acyl-CoAs, and bile acid CoA esters. Interestingly, despite the very high sequence homology, PTE-Ia and PTE-Ic showed different chain length specificities for straight-chain acyl-CoAs. Whereas PTE-Ia is a long-chain acyl-CoA thioesterase (highest activity with palmitoyl-CoA, C16-CoA), PTE-Ic is a medium-chain thioesterase (highest activity with decanoyl-CoA, C10-CoA) (Fig. 5).

**Molecular Characterization of Peroxisomal Thioesterases**

**FIG. 3.** Sequence alignment of PTE-Ia and PTE-Ic. Alignment of the amino acid sequences of PTE-Ia and PTE-Ic was performed using the Clustal X method. PTE-Ia 5':1 commences at the first methionine, whereas 5':2 commences at the second methionine. Individual exon boundaries are indicated. The catalytic triad consisting of a serine (corresponding to serine 232 in the PTE-Ic), a histidine (360), and an aspartic acid (326) residue is indicated with arrowheads. Both enzymes contain a carboxyl-terminal peroxisomal type 1 targeting signal of -AKL.

**FIG. 4.** PTE-Ia and PTE-Ic are peroxisomal proteins. Human skin fibroblasts were transfected with PTE-Ia-NT-GFP or PTE-Ic-NT-GFP and processed for immunofluorescence microscopy as outlined under "Experimental Procedures." The cellular localization of PTE-Ia and PTE-Ic was examined in control fibroblasts or Zellweger fibroblasts using a Tritc-labeled anti-GFP antibody. PTE-Ia in control fibroblasts (A), PTE-Ia in Zellweger fibroblasts (B), PTE-Ic in control fibroblasts (C), PTE-Ic in Zellweger fibroblasts (D).

**FIG. 5.** Kinetic characterization of recombinant PTE-Ia and PTE-Ic. Recombinant PTE-Ia and PTE-Ic were expressed in Escherichia coli as His-tagged proteins and purified by affinity chromatography as described under "Experimental Procedures." Acyl-CoA thioesterase activity was measured at 25 μM substrate concentration, with the addition of bovine serum albumin at a molar ratio of 1:4.5 of bovine serum albumin:acyl-CoA for C14-C20-CoAs in the case of PTE-Ia. The figure shows data from one representative experiment. The activity measurements were carried out on three different recombinant protein preparations for PTE-Ia and on two different recombinant protein preparations for PTE-Ic.
of the enzymes was active on bile acid-CoA esters (tested with choliol-CoA and chenodeoxycholiol-CoA). \(V_{\text{max}}\) and \(K_m\) values were calculated for those acyl-CoAs that were substrates for PTE-Ia and PTE-Ic (Table I). Further support for the difference in acyl-CoA chain length specificity stems from differences in the \(K_m\) values. The \(K_m\) values for PTE-Ia were generally much lower for long-chain acyl-CoAs than for PTE-Ic. Also, 4,8-dimethylnonanoyl-CoA, a metabolite in the \(\beta\)-oxidation of pristanic acid, was found to be a better substrate for PTE-Ic than for PTE-Ia (Table I).

We previously showed that mouse PTE-2, a peroxisomal acyl-CoA thioesterase that hydrolyzes a variety of CoA esters, is strongly inhibited by free CoASH (IC\(_{50}\) ~10–15 \(\mu\)M) (19), suggesting that PTE-2 regulates intraperoxisomal levels of free CoASH. We therefore tested the effect of CoASH on the acyl-CoA thioesterase activity of PTE-Ia and PTE-Ic. However, neither PTE-Ia nor PTE-Ic was markedly inhibited by CoASH. We therefore tested the effect of CoASH on the acyl-CoA thioesterase activity of PTE-Ia and PTE-Ic. However, neither PTE-Ia nor PTE-Ic was markedly inhibited by CoASH when tested at concentrations up to 500 \(\mu\)M (data not shown), suggesting that PTE-Ia and PTE-Ic are not involved in regulation of intraperoxisomal CoASH levels but rather regulate intraperoxisomal acyl-CoA levels.

**Tissue Expression**—The tissue expression of PTE-Ia and PTE-Ic at mRNA level was examined using RT-PCR in several tissues, with \(\beta\)-actin as a control (Fig. 6A). **PTE-Ia** and **PTE-Ic** showed a selective tissue expression, with **PTE-Ia** (both splice variants 5′:1 and 5′:2 amplified in the same PCR product) highly expressed in kidney and only weakly expressed in the other tissues examined. **PTE-Ic** is highly expressed in spleen, brain, testis, and proximal and distal intestine and weakly expressed in the other tissues. Both **PTE-Ia** and **PTE-Ic** are weakly expressed in liver from untreated mice but strongly increased at mRNA level by treatment of mice with WY-14,643 (Fig. 6A). A splice variant of **PTE-Ic** identified as the lower band on the gel in Fig. 6A (marked with an asterisk) was induced in liver by treatment with WY-14,643. **Western blot analysis** for PTE-Ia (5′:1) on muscle, heart, lung, brown adipose tissue, brain, and kidney showed no detectable protein expression in these tissues, except for liver with an induction seen in WY-14,643-treated liver. In kidney, no immunoreactive protein was seen (Fig. 6B) but was detectable in fasted kidney (where PTE-Ia 5′:1 may be strongly up-regulated). As mentioned above, in one of the splice variants for **PTE-Ia** (5′:2), the nucleotide encoding the first methionine is spliced out, producing a protein that is 11 amino acids shorter and that lacks the epitope recognized by the PTE-Ia antibody. Therefore, the antibody recognizes only the **PTE-Ia** 5′:1 splice variant. The lack of immunoreactive protein in muscle, heart, lung, brown adipose tissue, brain, and control kidney thus suggested that the **PTE-Ia** 5′:1 splice variant is expressed only in liver and that the high expression in kidney by RT-PCR (Fig. 6A) represents the 5′:2 splice variant. To test this possibility, we PCR-amplified the two splice variants in liver and kidney. As shown in Fig. 6C, the **PTE-Ia** 5′:2 splice variant is expressed in kidney, but not in liver. In contrast, the **PTE-Ia** 5′:1 splice variant encoding the longer PTE-Ia protein containing the antibody epitope could be PCR-amplified from liver, and this mRNA was increased in response to WY-14,643 treatment (data not shown).

**Regulation of PTE-Ia and PTE-Ic Expression by WY-14,643**

**Treatment and Fasting**—RT-PCR was carried out on total RNA from untreated and WY-14,643-treated liver from wild-type (+/+), PPAR\(\alpha\)-null (−/−) mice. Both **PTE-Ia** (splice variants 5′:1 and 5′:2) and **PTE-Ic** were highly induced by WY-14,643 treatment in wild-type mice but were not induced in the PPAR\(\alpha\)-null animals, showing that this induction is mediated via PPAR\(\alpha\) (Fig. 7A, upper panel). This induction was confirmed also at protein level by Western blot analysis for **PTE-Ia** (Fig. 7A, lower panel) and by Northern blot analysis for both **PTE-Ia** and **PTE-Ic** (data not shown). Northern blot analysis identified two mRNA transcripts of ~2.2 and 2.8 kb for **PTE-Ia**, which probably account for the difference in size of the two cDNA sequences for the 5′:1 and 5′:2 transcripts (~750 bp). Regulation of both **PTE-Ia** and **PTE-Ic** mRNA was also examined in liver, kidney, and heart from mice fasted for 24 h. RT-PCR showed that **PTE-Ia** was up-regulated by fasting in mouse liver in wild-type animals but not in the PPAR\(\alpha\)-null mice (Fig. 7B, upper panel). This PPAR\(\alpha\)-dependent up-regulation by fasting was also confirmed at protein level by Western blot analysis for **PTE-Ia** (Fig. 7B, lower panel).

Because **PTE-Ia** (both splice variants 5′:1 and 5′:2) showed a very high expression in kidney, we examined the regulation of **PTE-Ia** in this organ during the physiological condition of fasting. **PTE-Ia** mRNA expression was up-regulated by fasting in mouse kidney in wild-type animals, and again this up-regulation was abolished in the PPAR\(\alpha\)-null mice (Fig. 8). In contrast, expression of **PTE-Ic** showed no regulation by fasting either in
liver or kidney (Fig. 7B, upper panel and Fig. 8), and neither PTE-Ia nor PTE-Ic was regulated by fasting in heart (data not shown).

**DISCUSSION**

From previous work using molecular cloning and data base mining, we have now identified six highly homologous acyl-CoA thioesterase genes that are located in a cluster on mouse chromosome 12, of which four genes encode putative peroxisomal enzymes. These enzymes show \( \sim 40\% \) sequence identity to the bile acid-CoA:amino acid N-acyltransferase (BACAT) (26), an enzyme involved in the conjugation of bile acids to glycine or taurine (27–29). In addition, we have identified two other genes that are apparently located close to the gene for BACAT on mouse chromosome 4. These two genes also encode putative peroxisomal acyl-CoA thioesterases. This raised the question as to why there are so many peroxisomal acyl-CoA thioesterases in mouse. To address this issue, we have now cloned and characterized two of these genes that we named PTE-Ia and PTE-Ic and show that they are indeed peroxisomal enzymes with different functions based on their different acyl-CoA chain length specificities and tissue expression. Elucidation of the organization of the PTE-Ic gene shows that the open reading frame is encoded by three exons, similar to the PTE-Ia and other Type-I genes (17). In this gene family identified, the CTE-I and MTE-I show the highest degree of sequence identity to each other (>90%), whereas the PTE-Ia and PTE-Ic are the most similar peroxisomal thioesterases with a sequence identity of 82%. Most of the sequence differences between all six thioesterases are found in the third exon in the area where the active-site catalytic triad is located, which indicates that these thioesterases have different substrate specificities. Sequence analysis of PTE-Ia and PTE-Ic shows that there are two regions within the third exon that are less conserved, amino acids 257–280 of PTE-Ic (29% sequence identity) and amino acids 369–384 (22% sequence identity). The enzyme activity measurements showed that indeed these two enzymes have different substrate specificities, with PTE-Ia acting as a long-chain acyl-CoA thioesterase and PTE-Ic as a medium-chain acyl-CoA thioesterase.

Examination of tissue expression at mRNA level showed that these enzymes have a different and very selective tissue expression, with PTE-Ia being highly expressed in kidney and a
Molecular Characterization of Peroxisomal Thioesterases

highly up-regulated in liver by the hypolipidemic drug WY-14,643 at both mRNA and protein level, and this effect is mediated via PPARα. Although the highest PTE-Ia mRNA level was found in kidney, surprisingly, there was no detectable protein in kidney from untreated animals (Fig. 6B). In contrast, protein was readily detected in liver, although the mRNA levels were much lower in liver than in kidney (Fig. 6A). EST data base searches suggested that the 5'-untranslated region of PTE-Ia may be differently spliced, with one of the variants apparently splicing out the first start methionine, resulting in a truncated protein lacking the first 11 amino acids (5'/2) (corresponding to most of the epitope against which the PTE-Ia antibody was raised). Two EST sequences were found for this splice variant 5'/2 (accession numbers AW044864 and AI788456), both being derived from mouse kidney. Our results now show that PTE-Ia 5'/2 is expressed in kidney, but not in liver, whereas the longer PTE-Ia protein (containing the extra 11 amino acids) appears to be more liver-specific, resulting in different tissue expression of these two proteins. As yet it is not known what regulates the splicing within the different tissues, but the liver-specific expression of PTE-Ia 5'/1 may be regulated by liver-specific transcription factors. The function of the 11 extra amino acids at the amino-terminal of PTE-Ia is not clear. Sequence analysis did not indicate the presence of an amino-terminal type-2 peroxisomal targeting signal that is present in some peroxisomal matrix proteins (30) or the presence of a mitochondrial targeting signal. This was verified experimentally by expression of both the 5'/1 and 5'/2 PTE-Ia splice variants as carboxyl-terminal GFP fusion proteins in human skin fibroblasts. Both carboxyl-terminal PTE-Ia-GFP fusion proteins localized to the cytosol. In mammalian genomes, it now appears that alternative splicing of many genes results in different gene products (31) that may have different functions or show a distinct tissue expression. Indeed, a splice variant for PTE-Ic was also detected (marked with an asterisk) that splices out 118 bp of the open reading frame, resulting in a premature stop codon, which should result in an inactive protein that does not contain the active site histidine and aspartic acid residues. The expression of this splice variant also shows tissue selectivity and regulation by WY-14,643 (Figs. 6A and 7A).

Putative Functions for PTE-Ia and PTE-Ic in Peroxosomes— The selective tissue expression and variation in regulation indicates different functions for these two enzymes in peroxisomal lipid metabolism in different organs. Peroxisomes have important functions in the degradation of a broad spectrum of lipids, in particular lipids that are poorly metabolized by mitochondria (for reviews, see Refs. 2, 32, 33). The main function of peroxisomal β-oxidation is the metabolism of various carboxylic acids, for example, prostanoids and dicarboxylic fatty acids, into chain-shortened, more hydrophilic compounds that can be excreted in the urine as free carboxylic acids. Alternatively, peroxisomes chain-shorten long- and very long-chain acyl-CoAs, which may then be transported to mitochondria for further oxidation. The transport of fatty acids across the peroxisomal membrane is not fully understood, but based on studies in mammalian cells and yeast it appears that several mechanisms may operate in peroxisomes. Medium-chain fatty acids may be transported across the peroxisomal membrane as free acids and become activated to the corresponding CoA esters inside the peroxisome (34). In contrast, long-chain fatty acids, trihydroxycoprostanolic acid, and prostanoids become activated outside the peroxisome, followed by transport of the CoA esters across the peroxisomal membrane (for reviews, see Refs. 35, 36). Because medium-chain fatty acids are transported into the peroxisome, it is feasible to assume that they can also be

lower expression in the rest of the tissues examined, whereas PTE-Ic is highly expressed in spleen, brain, testis, and proximal and distal intestine and showed lower expression in the rest of the tissues. The expression of both of these enzymes is

![Fig. 7. Regulation of PTE-Ia and PTE-Ic in mouse liver by WY-14,643 treatment and fasting.](image)

![Fig. 8. Regulation of PTE-Ia and PTE-Ic by fasting in kidney.](image)
transported out of the peroxisome, for example in the case of chain-shortened fatty acids. It is hypothesized that PTE-Ia and PTE-Ic may be involved in this process by hydrolysis of long/medium-chain fatty acyl-CoA esters to the free fatty acid for transport out of the peroxisome to the mitochondria or endoplasmic reticulum for further β- or ω-oxidation. We also examined the activity of PTE-Ia and PTE-Ic toward the β-oxidation intermediate 3-OH palmitoyl-CoA, but these enzymes had very low activity toward this compound, suggesting that they can hydrolyze either the substrate or product of the β-oxidation pathway in peroxisomes but not intermediates in these pathways. The induction of both PTE-Ia and PTE-Ic by both peroxisome proliferators and/or fasting in liver and kidney is in line with an increase in β-oxidation activity in these organs during conditions that increase oxidation capacity.

It has been established that long/medium-chain fatty acids are transported to mitochondria as the carnitine ester for complete β-oxidation. Peroxisomes contain a carnitine octanoyltransferase that can potentially convert medium-chain acyl-CoA to the corresponding carnitine ester, which is required for transport into mitochondria. The physiological role of this carnitine octanoyl-transferase is not fully clear, but it was shown that this enzyme converts 4,8-dimethylnonanoyl-CoA, a metabolite of pristanic acid, to its corresponding carnitine ester, which is required for transport to mitochondria for further β-oxidation (37). In contrast, essentially no information is available concerning transport of free CoASH across the peroxisomal membrane. Two important functions can therefore be proposed for acyl-CoA thioesterases in peroxisomes: (i) to terminate chain shortening of various carboxylic acids to promote transport out of the peroxisome for transport to mitochondria for further oxidation (short-, medium-, and long-chain fatty acids) or for secretion into urine (dicarboxylic fatty acids and chain-shortened prostanoioids), and (ii) to regulate intraperoxisomal levels of acyl-CoA/CoASH.

In mouse six different Type-I acyl-CoA thioesterases have been identified, one cytosolic, one mitochondrial, and four peroxisomal acyl-CoA thioesterases (PTE-Ia-d) (Ref. 17, this study, and unpublished data). In addition, peroxisomes contain another structurally unrelated acyl-CoA thioesterase, called PTE-2. This enzyme was initially identified as an HIV-1 Nef-binding protein (21, 22) and subsequently further characterized as an acyl-CoA thioesterase in mouse, human, yeast, and rat (19, 20, 23, 38). Characterization of PTE-2 in mouse showed it to be the major acyl-CoA thioesterase in peroxisomes, hydrolyzing a broad range of acyl-CoA esters. The activity of recombinant PTE-2 is strongly regulated by CoASH (IC50 ~10 μM), suggesting that PTE-2 regulates intraperoxisomal CoA levels (19). Comparison of the acyl-CoA thioesterase activity in isolated peroxisomes with recombinant PTE-2 suggests that this enzyme is responsible for essentially all thioesterase activity except for the activity on medium- to long-chain acyl-CoAs that is induced in rat liver peroxisomes by peroxisome proliferator treatment (16). The findings reported in this study that PTE-Ia and PTE-Ic are peroxisome proliferator-induced long- and medium-chain acyl-CoA thioesterases therefore provide data to show that in principle PTE-2, PTE-Ia, and PTE-Ic are responsible for the peroxisomal acyl-CoA thioesterase activity covering C2–C22 acyl-CoAs. It is therefore likely that the remaining uncharacterized peroxisomal acyl-CoA thioesterases have activities toward other more specific substrates. Experiments are presently underway in our laboratory to examine this.

REFERENCES

1. Reddy, J. K., and Mannaeers, G. P. (1994) Annu. Rev. Nutr. 14, 343–370
2. Wanders, R. J. A., Vreken, P., Fedinandusse, S., Jansen, G. A., Waterham, H. R., van Roermund, C. W. T., and Van Grunsven, E. G. (2001) Biochem. Soc. Trans. 29, 250–255
3. Dezfaluzy, U., Kase, F. X., Alexson, S. E. H., and Bjerkhjem, I. (1991) J. Clin. Invest. 88, 978–984
4. Nemali, M. B., Usuda, N., Reddy, M. K., Oyasu, K., Hashimoto, T., Osumi, T., Rao, M. S., and Reddy, J. K. (1988) Cancer Res. 48, 5316–5324
5. Issermann, I., and Green, S. (1990) Nature 347, 645–650
6. Lee, S. S., Pineau, T., Drago, J., Lee, E. J., Owens, J. W., Kretz, D. L., Fernandez-Salgueiro, P. M., Westphal, H., and Gonzalez, F. J. (1996) Mol. Cell. Biol. 16, 3012–3022
7. Ferman, B. M., Chen, J., and Evans, R. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4312–4317
8. Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Kohle, C. S., Devchand, P., Wahl, W., Wilson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4318–4323
9. Göttlicher, M., Widmark, E., Li, Q., and Gustafson, J.-Å. (1999) Proc. Natl. Acad. Sci. U. S. A. 89, 4653–4657
10. Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., and Wahl, W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2160–2164
11. Kretz, D. L., Yonk, P., Costet, P., Bianchi, P., and Pineau, Y. (1998) J. Biol. Chem. 273, 31581–31589
12. Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B., and Wahli, W. (1999) J. Clin. Invest. 103, 1489–1498
13. Leone, T. C., Weinheimer, C. J., and Kelly, D. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7473–7478
14. Hunt, M. C., Lindquist, P. J., G. M., Peters, J. M., Gonzalez, F. J., Dezfaluzy, U., and Alexson, S. E. H. (2000) J. Lipid Res. 41, 814–823
15. Hunt, M. C., and Alexson, S. E. H. (2000) J. Lipid Res. 41, 99–130
16. Wilder, M., and Alexson, S. E. H. (1994) Eur. J. Biochem. 222, 865–871
17. Hunt, M. C., Nousiaian, S. E. B., Huttunen, M. K., U., Kori, Svensson, L. T., and Alexson, S. E. H. (1999) J. Biol. Chem. 274, 34317–34326
18. Jones, J. B., and Gould, S. J. (2000) Biochem. Biophys. Res. Commun. 275, 233–240
19. Hunt, M. C., Solaas, K., Kase, B. F., and Alexson, S. E. H. (2002) J. Biol. Chem. 277, 1128–1138
20. Jones, J. M., Nau, K., Geragthy, M. T., Erdmann, R., and Gould, S. J. (1999) J. Biol. Chem. 274, 9216–9223
21. Watanabe, H., Shiratori, T., Shoji, H., Miyatake, S., Okazaki, Y., Ikuta, K., Sato, T., and Saito, T. (1997) Biochem. Biophys. Res. Commun. 238, 234–239
22. Liu, L. X., Margottin, F., Le Gall, S., Schwartz, O., Selig, L., Benarous, R., and Hettema, E. H. (2000) Biochem. Biophys. Res. Commun. 279, 7478–7494
23. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
24. Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J., and Subramani, S. (1989) J. Cell Biol. 108, 1657–1664
25. Huffman, R., O’Byrne, J., Lindquist, P. J., Contreras, J. A., and Alexson, S. E. H. (2002) J. Biol. Chem. 277, 34324–34329
26. Falany, C. N., Fortinberry, H., Leiter, E. H., and Barnes, S. J. (1997) J. Biol. Chem. 272, 13779–13785
27. Tilton, G. B., Shockey, J. M., and Browse, J. (2003) J. Biol. Chem. 278, 3424–3431
28. Nakayama, H., Imamura, M., and Fujita, J. (1995) Biochem. Biophys. Res. Commun. 213, 208–213
29. Ofman, R., el Mrabet, L., Dacremont, G., Spijer, D., and Wanders, R. J. A. (2002) Biochem. Biophys. Res. Commun. 290, 629–634