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Identification of PTE2, a Human Peroxisomal Long-Chain Acyl-CoA Thioesterase

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Computer-based approaches identified *PTE2* as a candidate human peroxisomal acyl-CoA thioesterase gene. The *PTE2* gene product is highly similar to the rat cytosolic and mitochondrial thioesterases, CTE1 and MTE1, respectively, and terminates in a tripeptide sequence, serine-lysine-valine$_{COOH}$, that resembles the consensus sequence for type-1 peroxisomal targeting signals. PTE2 was targeted to peroxisomes and recombinant PTE2 showed intrinsic acyl-CoA thioesterase activity with a pH optimum of 8.5. A comparison of PTE2 and PTE1 thioesterase activities across multiple acyl-CoA substrates indicated that while PTE1 was most active on medium-chain acyl-CoAs, with little activity on long-chain acyl-CoAs, PTE2 displayed high activity on medium- and long-chain acyl-CoAs. The identification of PTE2 therefore offers an explanation for the observed long-chain acyl-CoA thioesterase activity of mammalian peroxisomes.

Key Words: **peroxisomes; fatty acid metabolism; thioesterase; coenzyme A; β-oxidation; α-oxidation.**

Acyl-CoA thioesterases catalyze the hydrolysis of acyl-CoAs to free fatty acids and coenzyme A. This activity has been well studied in the context of fatty acid synthesis, where the thioesterase component of fatty acid synthetase determines the length of fatty acids produced by terminating the synthesis cycle (1). Recently, new thioesterase activities have been identified that are not involved in fatty acid synthesis (1–11). Most of these thioesterases are compartmentalized within mitochondria and peroxisomes, organelles involved in fatty acid degradation. Furthermore, many of these thioesterases are upregulated by peroxisome proliferating agents, compounds that stimulate the expression of many enzymes involved in fatty acid oxidative pathways (12).

Studies on the thioesterase activity of isolated rat peroxisomes showed that these organelles exhibit acyl-CoA thioesterase activity on a broad spectrum of substrates (5). Highest activity was found with acyl-CoAs of chain lengths ranging from 8 to 14 carbons, while acyl-CoAs with carbon chains of 2 to 6 carbons or 16 to 20 carbons showed approximately half of this level of activity. In addition, peroxisomal proliferating agents were observed to selectively stimulate peroxisomal acyl-CoA thioesterase activity towards substrates with chain lengths of 10 to 14 carbons. These findings led the authors to propose the existence of up to three mammalian peroxisomal thioesterases. In another report, two distinct peroxisomal thioesterase activity peaks were resolved by size exclusion chromatography (3). One peak corresponded to an enzyme of approximately 35 kDa and one to a larger enzyme, approximately 60 kDa. PTE1, a 35 kDa peroxisomal acyl-CoA thioesterase, is primarily active on acyl CoAs with chain lengths ranging from 10 to 14 carbons (8, 10). It has little or no activity on acyl CoAs longer than 14 carbons. The presence of significant long-chain acyl-CoA thioesterase activities in peroxisomes is therefore not explained by the activity of PTE1. Rather, these findings suggest the existence of peroxisomal thioesterases in addition to PTE1.

Here we report the result of a computer-based search for human genes that might encode additional peroxisomal thioesterases. We identified *PTE2*, a gene similar to previously reported rat cytosolic and mitochondrial thioesterases (CTE1 (7) and MTE1 (4), respectively). *PTE2* encodes a peroxisomal protein with intrinsic acyl-CoA thioesterase activity. This enzyme shows maximal activity at pH 8.5, approximately the pH of the peroxisome lumen (13), and shows significant activity on long-chain substrates, providing a structural basis for long-chain acyl-CoA thioesterase activity in mammalian peroxisomes.

MATERIALS AND METHODS

Plasmids. The *PTE2* open reading frame (ORF) was amplified from a cDNA clone (GenBank Acc. No. W39420, IMAGE:322484) using the primers 5′-CCCGTCGACGATGGCGGCGACGCTGATC-

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CTG-3′ and 5′-CCCGCGGCCGCTTACACTTTTGATGGGATTGTC-CCCTC-3′. The PCR product was digested with *SalI* and *NotI* (sites underlined) and cloned into the *SalI* and *NotI* sites of pT7-His_6 (14), a modified form of the pET28a expression vector (Novagen), to make pT7-His_6/PTE2. The *PTE2* ORF was excised from pT7-His_6/PTE2 by digestion with *SalI* and *NotI* and inserted into the *XhoI* and *NotI* sites of pcDNA3-Nmyc (10) to make pcDNA3-Nmyc/PTE2. This vector is designed to express proteins in fusion with an amino terminal *c-myc* epitope. All bacterial plasmid manipulations were performed in the *Escherichia coli* strain DH10B (15).

Transfections, immunofluorescence, antibodies, and fluorescence microscopy. Indirect immunofluorescence studies were done in normal human skin fibroblasts (GM5756-T) or the Zellweger syndrome cell line, PBD100. The PBD100 cell line is homozygous for an inactivating mutation in *PEX10*, and has been described previously (16). Cells were cultured and transfected as described (17). After electroporation, cells were plated on cover glasses and incubated for two days. Cells were then fixed and processed for indirect immunofluorescence as described (17). Permeabilization was normally performed using 1% Triton X-100, which permeabilizes both the plasma and peroxisome membranes. For selective permeabilization of only the plasma membrane, 25 μg/ml digitonin was used. The monoclonal anti-myc antibody used was derived from the tissue culture supernatant of the mouse hybridoma cell line 1-9E10 (Roche Molecular Biochemicals). Sheep antibodies against PMP70 were made against a peptide which corresponds to the cytosolic carboxy-terminal domain of this peroxisomal protein. Fluorescent secondary antibodies were obtained from commercial sources.

Expression and purification of recombinant proteins. A 50 ml culture of BL21/DE3 (Novagen) cells harboring the plasmid pT7-His_6/PTE2 was grown overnight at 37°C in Luria broth supplemented with 25 μg/ml kanamycin. This culture was diluted to 1 L with 2YT medium supplemented with 25 μg/ml kanamycin and grown to an optical density (OD_{600}) of approximately 0.4. The culture was then cooled to room temperature, induced with 1 mM IPTG, and allowed to grow overnight with vigorous shaking. Cells were harvested, washed once with Luria broth, and resuspended in 25 ml binding buffer (20 mM NaP_i pH 7.8, 500 mM NaCl, 5 mM benzamidine HCl) containing 0.4 mg/ml lysozyme, 8 μg/ml DNAse, and 8 μg/ml RNAse. Cells were incubated on ice for 20 min, frozen in liquid nitrogen, thawed, and lysed by mixing. The lysate was cleared by centrifugation at 25,000*g* for 30 min. The resulting supernatant was diluted to 50 ml in binding buffer and loaded onto a Chelating Sepharose Fast-Flow column (Pharmacia) that had been previously charged with NiCl. The column was washed with 10 bed volumes of binding buffer followed by five volumes of wash buffer (20 mM NaP_i pH 6.0, 500 mM NaCl, 5 mM benzamidine HCl), and the bound proteins were eluted using sequential 5 ml steps of 50 mM imidazole, 200 mM imidazole, 300 mM imidazole, and 500 mM imidazole in wash buffer. Fractions were analyzed by SDS PAGE, and those containing highly purified HIS_6/PTE2 were pooled and precipitated by the addition of $(NH4)_2SO_4$ for storage at −80°C.

Enzyme assays. Stored recombinant PTE2 was resuspended in 25% glycerol, 50 mM KP_i pH 6.0 (higher pH resulted in precipitation of concentrated PTE2) and used immediately. Assays of PTE proteins were performed in assay buffer (50 mM KPi pH 8.5, 0.2 mg/ml BSA, 100 μM 5,5′-dithiobis (2-nitrobenzoic acid)) containing the appropriate acyl CoA substrate. For activity studies across a range of pH, a modified assay buffer (20 mM KP_i, 20 mM Tris HCl, 20 mM CAPS, 0.2 mg/ml BSA, 100 μM 5,5′-dithiobis (2-nitrobenzoic acid)) was used. Reactions were started by the addition of PTE proteins and PTE activities were determined by monitoring the change in A_{412} with time. All assays were done at room temperature (20°C). One unit of thioesterase activity is defined as the amount required to hydrolyze one micromole of acyl-CoA per minute.

RESULTS

PTE2 is a member of a family of highly similar thioesterase genes. The inability of PTE1 to explain the reported long-chain acyl-CoA thioesterase activities in mammalian peroxisomes led us to search the database of expressed sequence tags (ESTs) for additional peroxisomal thioesterases. Specifically, we searched for genes other than PTE1 that could encode proteins that were similar to known thioesterases and contained a peroxisomal targeting signal 1 (PTS1) motif. We identified one candidate human cDNA (GenBank Accession No. W39420 (IMAGE:322484)) that appeared to have these properties. This 1493-bp cDNA contained a 1266-bp open reading frame that terminated in the near-consensus PTS1, serine-lysine-$valine_{COOH}$ (Fig. 1). The predicted gene product of the *PTE2* cDNA is highly similar to multiple thioesterases previously identified in rodent species, as well as rat and human bile acid-amino acid N-acyltransferases (18, 19) (Fig. 2A). The deduced amino acid sequence of human PTE2 is 76.0% identical to rat CTE1 and 72.3% identical to rat MTE1. Human PTE2 also contains the GXSXG motif (amino acids 230–234) that is thought to contain the active sight serine of thioesterases in this family. However, PTE2 is only 19.5% identical to human PTE1, suggesting that these genes are evolutionarily distant. Figure 2B shows a phylogenetic tree of a sample of thioesterases and related genes. This family of thioesterases extends beyond mammalian species. Four predicted proteins in the *Caenorhabditis elegans* database are highly similar to PTE2. Interestingly, all four of these predicted proteins terminate in a consensus PTS1, indicating that the presence of multiple peroxisomal thioesterases is a common feature of higher eukaryotes.

PTE2 is imported into the peroxisome lumen. The PTE2 ORF contains a near-consensus PTS1 at its C-terminus. To test whether PTE2 is actually peroxisomal, we modified the PTE2 open reading frame to contain an 18-amino acid myc tag at its 5′ end. A plasmid designed to express this myc/PTE2 fusion, pcDNA3-Nmyc/*PTE2*, was introduced into human skin fibroblasts by electroporation. Indirect immunofluorescence experiments revealed that Nmyc/PTE2 was imported into peroxisomes (Fig. 3A), as determined by colocalization with the peroxisomal marker protein PMP70 (Fig. 3B). However, in all cells expressing Nmyc/PTE2, some PTE2 was also visible in the cytoplasm. Some cytoplasmic labeling is common when overexpressing peroxisomal matrix proteins. However, for other peroxisomal matrix proteins that we have studied, including PTE1 (10), PECI (14), PDCR (20), HAOX1, HAOX2, and HAOX3 (21), the majority of expressing cells show only peroxisomal staining.

The same transfected cells were also processed for immunofluorescence using digitonin to achieve differ-

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                                                                caatggagcctg  -73
aagagttcggcgcagttcctggggtctccacagctgaggcaggttggtcagatcattagggttcctgctcgg  - 1

ATGGCGGCGACGCTGATCCTGGAGCCTGCGGGCCGCTGCTGCTGGGACGAACCGGTGCGAATCGCCGTGCGC   72
 M  A  A  T  L  I  L  E  P  A  G  R  C  C  W  D  E  P  V  R  I  A  V  R    24

GGCCTAGCCCCGGAGCAGCCGGTCACGCTGCGCGCGTCCCTGCGCGACGAGAAGGGCGCGCTTTTCCAGGCC  144
 G  L  A  P  E  Q  P  V  T  L  R  A  S  L  R  D  E  K  G  A  L  F  Q  A    48

CACGCGCGCTACCGCGCCGACACTCTTGGCGAGCTGGACCTGGAGCGCGCGCCCGCGCTGGGCGGCAGCTTC  216
 H  A  R  Y  R  A  D  T  L  G  E  L  D  L  E  R  A  P  A  L  G  G  S  F    72

GCGGGGCTTGAGCCCATGGGGCTGCTCTGGGCCTTGGAGCCCGAGAAACCTTTGGTGCGGCTGGTGAAGCGC  288
 A  G  L  E  P  M  G  L  L  W  A  L  E  P  E  K  P  L  V  R  L  V  K  R    96

GACGTGCGAACGCCCTTGGCCGTGGAGCTGGAGGTGCTGGATGGCCACGACCCCGACCCCGGGCGGCTGCTG  360
 D  V  R  T  P  L  A  V  E  L  E  V  L  D  G  H  D  P  D  P  G  R  L  L   120

TGCCAGACGCGGCACGAGCGCTACTTCCTCCCGCCCGGGGTGCGGCGCGAGCCGGTGCGCGTGGGCCGGGTG  432
 C  Q  T  R  H  E  R  Y  F  L  P  P  G  V  R  R  E  P  V  R  V  G  R  V   144

CGAGGCACGCTCTTCCTGCCGCCAGAACCTGGGCCCTTTCCTGGGATTGTGGACATGTTCGGAACTGGAGGT  504
 R  G  T  L  F  L  P  P  E  P  G  P  F  P  G  I  V  D  M  F  G  T  G  G   168

GGCCTGCTGGAGTATCGGGCTAGTCTGCTGGCTGGGAAGGGTTTTGCTGTGATGGCTCTGGCTTATTATAAC  576
 G  L  L  E  Y  R  A  S  L  L  A  G  K  G  F  A  V  M  A  L  A  Y  Y  N   192

TATGAAGACCTCCCCAAGACCATGGAGACGCTCCATCTGGAGTACTTTGAAGAAGCCATGAACTACTTGCTC  648
 Y  E  D  L  P  K  T  M  E  T  L  H  L  E  Y  F  E  E  A  M  N  Y  L  L   216

AGTCATCCCGAGGTAAAAGGTCCAGGAGTTGGGCTGCTTGGAATTTCCAAAGGGGGTGAGCTCTGCCTTTCC  720
 S  H  P  E  V  K  G  P  G  V  G  L  L  G  I  S  K  G  G  E  L  C  L  S   240

ATGGCCTCTTTCCTGAAGGGCATCACGGCTGCTGTCGTCATCAACGGCTCTGTGGCCAATGTTGGGGGAACC  792
 M  A  S  F  L  K  G  I  T  A  A  V  V  I  N  G  S  V  A  N  V  G  G  T   264

TTACGCTACAAGGGCGAGACCCTGCCCCCTGTGGGCGTCAACAGAAATCGCATCAAGGTGACCAAAGATGGC  864
 L  R  Y  K  G  E  T  L  P  P  V  G  V  N  R  N  R  I  K  V  T  K  D  G   288

TATGCAGACATTGTGGATGTCCTGAACAGCCCTTTGGAAGGACCTGACCAGAAGAGCTTCATTCCTGTGGAA  936
 Y  A  D  I  V  D  V  L  N  S  P  L  E  G  P  D  Q  K  S  F  I  P  V  E   312

AGGGCAGAGAGCACCTTCCTGTTCCTGGTAGGTCAGGATGACCACAACTGGAAGAGTGAGTTCTATGCTAAT 1008
 R  A  E  S  T  F  L  F  L  V  G  Q  D  D  H  N  W  K  S  E  F  Y  A  N   336

GAGGCCTGTAAACGCTTGCAGGCCCATGGGAGGAGAAAGCCCCAGATCATCTGTTACCCAGAGACAGGGCAC 1080
 E  A  C  K  R  L  Q  A  H  G  R  R  K  P  Q  I  I  C  Y  P  E  T  G  H   360

TATATTGAGCCTCCTTACTTCCCCCTGTGTCGGGCTTCCCTGCATGCCTTGGTGGGCAGTCCTATTATCTGG 1152
 Y  I  E  P  P  Y  F  P  L  C  R  A  S  L  H  A  L  V  G  S  P  I  I  W   384

GGAGGGGAGCCCAGGGCTCATGCCATGGCTCAGGTGGATGCTTGGAAACAACTCCAGACTTTCTTCCACAAA 1224
 G  G  E  P  R  A  H  A  M  A  Q  V  D  A  W  K  Q  L  Q  T  F  F  H  K   408

CACTTGGGTGGCCGCGAGGGGACAATCCCATCAAAAGTGTAAattttatttgatcatgtagcctctctgttg 1296
 H  L  G  G  R  E  G  T  I  P  S  K  V  *                                 421

ctaatctctcctggaaacatctgccacatttagtgtgtgtatgtgtattcattcttttgtttttaataacta 1368
aagttttttcccctcattattaaaatgaatttaccagtaag                                 1409
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FIG. 1. Nucleotide and predicted protein sequence of the PTE2 cDNA. The 1266-bp open reading frame terminates in the near-consensus type 1 peroxisomal targeting sequence, serine-lysine-valine$_{COOH}$ (underlined), and is predicted to encode an acidic protein (p*I* 6.51) with a mass of 46.3 kDa.

A

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1   MAA-----------------------------------------------TLILEPAGRCCWDEPV HsPTE2
1   M-------------------------------------------------EATLSLGPAGRSCWDEPL RnCTE1
1   MVASSFAVLRASRLCQWGWKSWTQLSGPPPLSTGGRTTFARTNATLSLEPGSRSCWDEPL RnMTE1
1   MA----------------------KLTAVP---------------LSALV----------DEPV RnKAN1
1   MI----------------------QLTATP---------------VSALV----------DEPV HsBAT

20  RIAVRGLAPEQPVTLRASLRDEKGALFQAHARYRADTLGELDLERAPALGGSFAGLEPMG HsPTE2
20  SITVRGLVPEQPVTLRAALRDEKGALFRARALYRADAHGELDLARAPALGGSFTGLEPMG RnCTE1
61  SITVRGLAPEQPVTLRAALRDEKGALFRAHARYRADAGGELDLARAPALGGSFTGLEPMG RnMTE1
18  HIRVTGLTPFQVVCLQASLKDDKGNLFNSQAFYRASEVGEVDLERDSSLGGDYMGVHPMG RnKAN1
18  HIRATGLIPFQMVSFQASLEDENGDMFYSQAHYRANEFGEVDLNHASSLGGDYMGVHPMG HsBAT

80  LLWALEPEKPLVRLVKRDV-RTPLAVELEVLDGHDPDPGRLLCQTRH----ERYFLPPGV HsPTE2
80  LLWAMEPERPFWRLVKRDV-QTPFVVELEVLDGHKPDGGRLLARAVH----ERHFMAPGV RnCTE1
121 LIWAMEPERPLWRLVKRDV-QKPYVVELEVLDGHEPDGGQRLAQAVH----ERHFMAPGV RnMTE1
78  LFWSMKPEKLLTRLVKRDVMNRPHKVHIKLCHPYFPVEGKVISSSLDSLILERWYMAPGV RnKAN1
78  LFWSLKPEKLLTRLLKRDVMNRPFQVQVKLYDLELIVNNKVASAPKASLTLERWYVAPGV HsBAT

135 RREPVRVGRVRGTLFLPPEPGPFPGIVDMFGTGGGLLEYRASLLAGKGFAVMALAYYNYE HsPTE2
135 RRVPVREGRVRATLFLPPEPGPFPGIINLFGVGGGLLEYRASLLAGKGFAVMALAYYNYD RnCTE1
176 RRVPVRDGRVRATLFLPPEPGPFPEIIDLFGVGGGLLEYRASLLAGKGFAVMALAYYNYD RnMTE1
138 TRIHVKEGRIRGALFLPPGEGPFPGVIDLFGGAGGLFEFRASLLASHGFATLALAYWGYD RnKAN1
138 TRIKVREGRLRGALFLPPGEGLFPGVIDLFGGLGGLLEFRASLLASRGFASLALAYHNYE HsBAT

195 DLPKTMETLHLEYFEEAMNYLLSHPEVKGPGVGLLGISKGGELCLSMASFLKGITAAVVI HsPTE2
195 DLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGGELGLAMASFLKGITAAVVI RnCTE1
236 DLPKTMETMRIEYFEEAVNYLRGHPEVKGPGIGLLGISKGGELGLAMASFLKGITAAVVI RnMTE1
198 DLPSRLEKVDLEYFEEGVEFLLRHPKVLGPGVGILSVCIGAEIGLSMAINLKQITATVLI RnKAN1
198 DLPRKPEVTDLEYFEEAANFLLRHPKVFGSGVGVVSVCQGVQIGLSMAIYLKQVTATVLI HsBAT

255 NGSVANVGEPYGYKGETLPPVGVNRNRIKVTKDGYADIVDVLNSPLEGPDQKSFIPVERA HsPTE2
255 NGSVAAVGNTICYKDETIPPVTILRNQVKMTKDGLKDVVDALQSPL--VEQKSFIPVERS RnCTE1
296 NGSVAAVGNTVCYKDETIPPVSLLRDKVKMTKDGLLDVVEALQSPL--VDKKSFIPVERS RnMTE1
258 NGPNFVSSNPHVYRGKVFQPTPCSEEFVTTNALGLVEFYRTFEETADKDSKYCF-PIEKA RnKAN1
258 NGTNFPFGIPQVYHGQIHQPLPHSAQLISTNALGLLELYRTFETTQVGASQYLF-PIEEA HsBAT

315 ESTFLFLVGQDDHNWKSEFYANEACKRLQAHGRRKPQIICYPETGHYIEPPYFPLCRASL HsPTE2
313 DTTFLFLVGQDDHNWKSEFYANEISKRLQAHGKEKPQIICYPEAGHYIEPPYFPLCSAGM RnCTE1
354 DTTFLFLVGQDDHNWKSEFYARFASKRLQAHGKEKPQIICYPEAGHYIEPPYFPLCSAGM RnMTE1
317 HGHFLFVVGEDDKNLNSKVHAKQAIAQLMKSGKKNWTLLSYPGAGHLIEPPYSPLCSASR RnKAN1
317 QGQFLFIVGEGDKTINSKAHAEQAIGQLKRHGKNNWTLLSYPGAGHLIEPPYSPLCCAST HsBAT

375 HALVGSPIIWGGEPRAHAMAQVDAWKQLQTFFHKHLGGREGTIPSKV HsPTE2
373 HLLVGANITFGGEPKPHSVAQLDAWQQLQTFFHKQLGGKSHGVSPKI RnCTE1
414 HLLVGANITFGGEPKPHSVAQLDAWQQLQTFFHKQLSGKS RnMTE1
377 MPFVIPSINWGGEVIPHAAAQEHSWKEIQKFLKQHL---NPGFNSQL RnKAN1
377 THDL--RLHWGGEVIPHAAAQEHAWKEIQRFLRKHL---IPDVTSQL HsBAT
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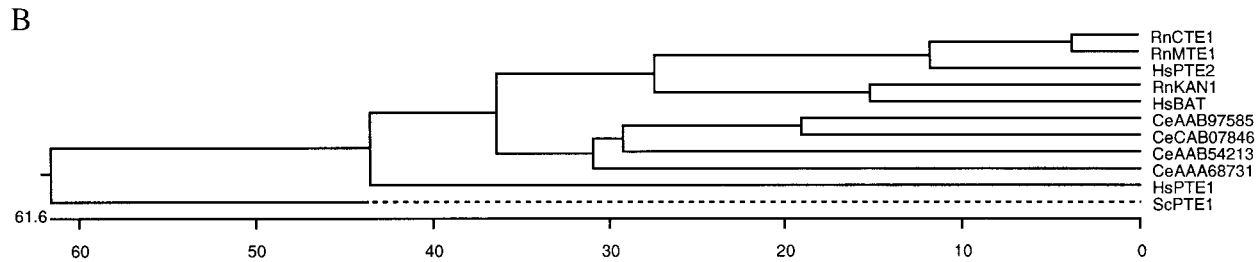
B



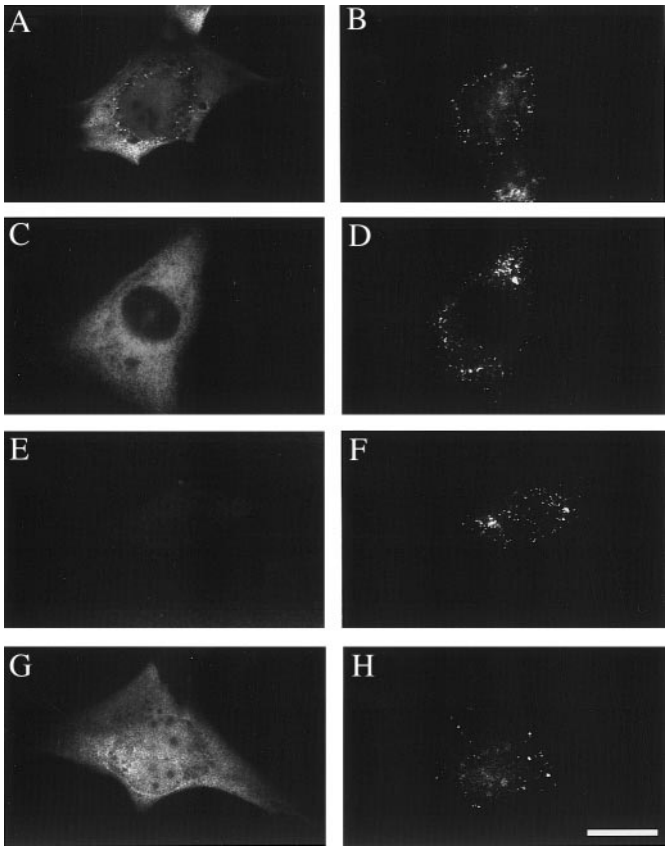


FIG. 3. PTE2 is a peroxisomal matrix protein. Human skin fibroblasts expressing Nmyc/PTE2 were processed for indirect immunofluorescence by fixing cells and permeabilizing with 1% Triton X-100. The distribution of Nmyc/PTE2 was examined using anti-myc (A) and anti-PMP70 (B) antibodies. Additional cells expressing Nmyc/PTE2 were permeabilized with 25 μg/ml digitonin and examined again using anti-myc (C), anti-PTE1 (E), and anti-PMP70 antibodies (D, F). The distribution of Nmyc/PTE2 was also examined in the *PEX10*-deficient cell line PBD100 using anti-myc (G) and anti-PMP70 (H) antibodies. Scale bar, 25 μm.

ential permeabiliztion. Under these conditions, the plasma membrane is permeabilized while the peroxisome membrane is left intact. Indirect immunofluorescent labeling of Nmyc/PTE2 in digitonin-permeabilized cells showed only cytoplasmic staining (Fig. 3C). Peroxisomes were readily detected using PMP70 antibodies that recognize the cytosolic carboxy-terminal domain of this peroxisomal membrane protein (Fig. 3D). These results demonstrate that the peroxisomal PTE2 had been imported into the peroxisome lumen. As a control, we also studied this same transfected cell population with antibodies raised against PTE1, a known peroxisomal matrix protein (10). Only background staining is observed for PTE1 in digitonin-permeabilized cells (Figs. 3E and 3F), demonstrating the efficacy of the differential permeabilization technique.

As an independent test of whether PTE2 is imported into the peroxisome matrix, we expressed Nmyc/PTE2 in a Zellweger syndrome skin fibroblast cell line, PBD100. The PBD100 cell line is homozygous for an inactivating mutation in *PEX10* and is unable to import peroxisomal matrix proteins, although it does contain numerous peroxisomes and imports peroxisomal membrane proteins normally (16). Nmyc/PTE2 was seen only in the cytosol of PBD100 cells, even though these cells contained numerous PMP70-containing peroxisomes (Figs. 3G and 3H).

Recombinant PTE2 shows intrinsic long-chain acyl-CoA thioesterase activity. The PTE2 open reading frame was modified to include 6 histidine codons at its 5′ end. The encoded fusion protein, His_6/PTE2, was expressed in bacteria and purified by nickel affinity chromatography (Fig. 4A). Recombinant His_6/PTE2 showed acyl-CoA thioesterase activity towards a broad range of acyl-CoAs, but appeared to be most active on myristoyl-CoA. Figure 4B shows PTE2 activities at different myristoyl-CoA concentrations. PTE2 did not exhibit a Michaelis-Menten type kinetic profile, but rather showed inhibition of thioesterase activity above 30 μM myristoyl-CoA. The maximum specific activity of PTE2 on myristoyl-CoA was 0.75 U/mg. PTE2 activity was also measured across a broad pH range, and was found to be optimal at pH 8.5 (Fig. 5) with a sharp decline in activity at either higher or lower pH values. The thioesterase activity of PTE2 was compared to that of PTE1 using a variety of acyl-CoA substrates (Fig. 6). As noted previously (8), PTE1 showed highest activity on the medium-chain acyl-CoAs, lower activity on shorter chain acyl-CoAs, and very little activity on acyl-CoAs with chains longer than fourteen carbons. PTE2, in contrast, was most active on myristoyl-CoA but also showed high activity on the longer chain acyl-CoAs palmitoyl-CoA, stearoyl-CoA, and arachidoyl-CoA. Both PTE1 and PTE2 showed low activity towards the short branched-chain substrate isobutyryl-CoA.

DISCUSSION

Peroxisomes serve multiple cellular metabolic functions, many of which involve lipid degradation, modi-

FIG. 2. PTE2 is a member of a family of thioesterase enzymes. (A) Alignment of the amino acid sequences of PTE2 and four related enzymes. RnCTE1, rat cytosolic thioesterase; RnMTE1, rat mitochondrial thioesterase; RnKAN1, rat bile acid–amino acid *N*-acetyltransferase; HsBAT, human bile acid–amino acid *N*-acetyltransferase. Residues identical in three or more proteins are boxed. (B) Phylogenetic tree of mulitple thioesterases. HsPTE1, human peroxisomal thioesterase 1; ScPTE1, yeast peroxisomal thioesterase 1; CeCAB07846, CeAAB97585, CeAAB54213, CeAAA68731: four *Caenorhabditis elegans* predicted proteins (number codes are GenBank accession numbers). Sequence alignment and phylogenetic tree were constructed using DNASTAR (Madison, WI) and the PAM 250 matrix.

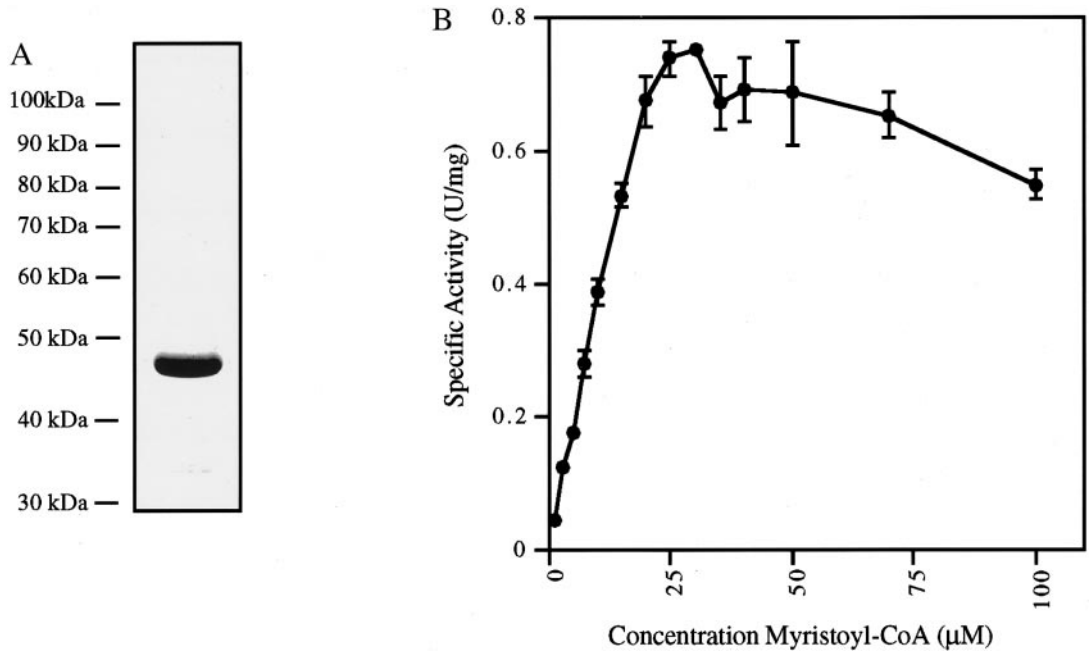


FIG. 4. Recombinant PTE2 has intrinsic acyl-CoA thioesterase activity. (A) Purified His_6/PTE2 stained with Coomassie blue. (B) Acyl-CoA thioesterase activity of His_6/PTE2 at various myristoyl-CoA concentrations. Activity was determined using the free thiol indicator 5,5′-dithiobis(2-nitrobenzoic acid) by following the increase in A_{412} with time. Data are the mean ± standard deviation of four trials.

fication, and synthesis (22). Many pathways that metabolize or utilize fatty acids act only on acyl-CoA substrates rather than free fatty acids. Therefore, one might expect that acyl-CoA thioesterase activities would be inhibitory to peroxisomal metabolic processes. However, numerous studies have reported the presence of multiple peroxisomal acyl-CoA thioesterase activities in this organelle. Biochemical studies have shown that isolated rat peroxisomes display acyl-CoA thioesterase activity towards a wide range of substrates, with highest activity on medium-chain acyl-CoAs (chains of 8 to 14 carbons) and approximately half of this activity on short-chain acyl CoAs (chains of 2 to 6 carbons) and long-chain acyl CoAs (chains of 16 to 20 carbons) (5). Two distinct peroxisomal thioesterase activities, corresponding to enzymes of approximately 35 kDa and 60 kDa, have been resolved by size exclusion chromatography (3), and purification of peroxisomal myristoyl-CoA thioesterase activity led to the identification of an approximately 46 kDa polypeptide (5).

As part of an effort to understand the metabolic roles of peroxisomal thioesterases, we have attempted to elucidate the structural bases for these enzyme activities. Recently we reported the identification of PTE1, a peroxisomal thioesterase of yeast and humans (10). Human PTE1 is active primarily on medium chain substrates (8 to 14 carbons) with very little activity on acyl CoAs with chains longer than 14 carbons. The activity of PTE1 therefore does not explain the existence of long-chain acyl-CoA thioesterase activity in isolated peroxisomes, suggesting the existence of one or more additional peroxisomal thioesterases with long chain acyl CoA thioesterase activity.

In this report we identify PTE2, a second human peroxisomal thioesterase. *PTE2* was identified in a computer based search for genes similar to known thioesterases. The PTE2 ORF terminates in a near-consensus peroxisome targeting signal type 1, serine-lysine-valine$_{COOH}$, and encodes a protein that is at least partly targeted to peroxisomes. Recombinant PTE2 showed intrinsic acyl-CoA thioesterase activity and,

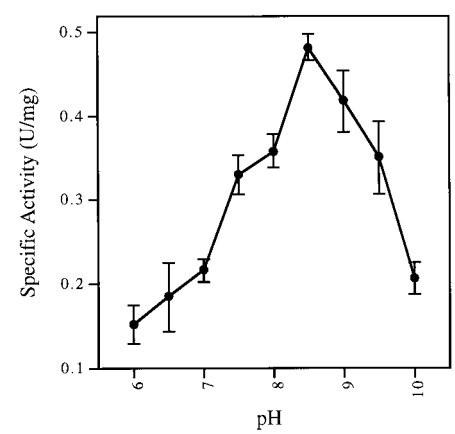


FIG. 5. Recombinant PTE2 shows optimal activity at pH 8.5. The acyl-CoA thioestease activity of His_6/PTE2 was determined across a range of pH. 100 μM of myristoyl-CoA was used as substrate. Data are the mean ± standard deviation of 3 trials.

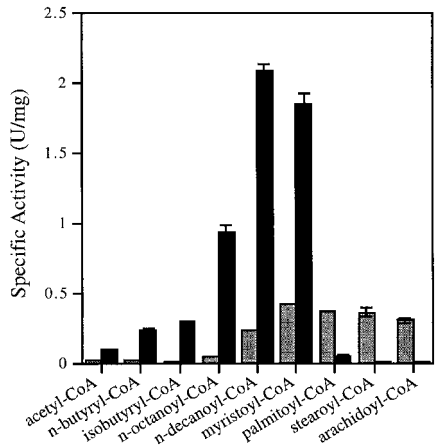


FIG. 6. Comparison of PTE1 and PTE2 activities on a variety of acyl-CoA substrates. Specific activities of PTE1 and PTE2 toward acyl-CoAs of varying carbon chain lengths were determined using 100 μM of each substrate. Dark bars, PTE1; light bars, PTE2. Data are the mean ± standard deviation of three trials.

most importantly, was highly active on the long chian substrates palmitoyl-CoA, stearoyl-CoA, and arachidoyl-CoA. The identification of PTE2 therefore provides an explanation for the observed long chain acyl CoA activity of peroxisomes. PTE2 showed optimal activity at pH 8.5, a pH similar to that of the peroxisome lumen (approximately pH 8.0), suggesting that this enzyme may be optimally suited for the peroxisomal environment (13). In addition, thioesterases similar to PTE2 are serine thioesterases that work via a nucleophilic attack mechanism and may be most active in a basic pH environment (4).

PTE2 shares only limited homology with PTE1 and is instead very similar to the rat cytosolic thioesterase, CTE1 (7). These enzymes appear to belong to a family of thioesterases that includes the mitochondrial thioesterase MTE1 and bile acid-amino acid N-acetyltransferases (4, 18, 19). In addition, the *Caenorhabditis elegans* genome database contains four predicted proteins similar to PTE2, all four of which end in a consensus or near-consensus PTS1. These genes likely represent four peroxisomal thioesterases, suggesting that the presence of multiple peroxisomal thioesterases is a conserved feature of higher eukaryotes.

The variant of the PTS1 found in PTE2, SKV_{COOH}, has not previously been reported to function in human cells. However, our studies of PTE2 distribution showed that in normal human fibroblasts this enzyme is indeed targeted to peroxisomes. Differential permeabilization studies showed that PTE2 was imported into the peroxisome lumen. Furthermore, our finding that PTE2 is not imported into peroxiosmes in Zellweger syndrome patient cells provides independent evidence that PTE2 is a peroxisomal matrix enzyme.

While there is no doubt that PTE2 is peroxisomal, it was not imported efficiently. All cells showing peroxisomal staining for Nmyc/PTE2 also showed cytosolic staining for this fusion protein. It is formally possible that the cytosolic accumulation of Nmyc/PTE2 may be an artifact of overexpression or the presence of the eighteen amino acid myc tag at the PTE2 N-terminus. However, we do not think that this is the case. Past experience with multiple other peroxisomal matrix enzymes such as PTE1 (10), PECI (14), PDCR (20), HAOX1, HAOX2, and HAOX3 (21), has shown us that overexpressed and tagged proteins are usually imported quite efficiently. Instead, we believe that the PTS1 of this protein may be so inefficient as to preclude the complete incorporation of PTE2 into peroxisomes, resulting in a partial cytosolic distribution for this enzyme. Some evidence to support the concept that SKV_{COOH} may be an inefficient peroxisomal targeting signal may be found in the initial work on the PTS1 of firefly luciferase, where the SKV_{COOH} motif did not function as an effective targeting signal (23). A steady-state partitioning of proteins between the peroxisome and cytosol has also been observed for other peroxisomal matrix enzymes, including malonyl-CoA decarboxylase (MCD) (24) and isocitrate dehydrogenase (PICD) (25). Interestingly, the rat cytosolic thioesterase, CTE1, also terminates in a near consensus PTS1, PKI_{COOH}, and is highly similar to PTE2, raising the possibility that these two enzymes may be orthologous.

The existence of multiple peroxisomal thioesterases indicates that thioesterase enzymes play an important role in the metabolic functions of this organelle. However, what that role is remains unknown. Our finding that yeast PTE1 is upregulated by fatty acids and is required for efficient yeast growth on fatty acids led us to propose a model in which PTE1 plays an auxiliary role in fatty acid β-oxidation (10). In this model, peroxisomal thioesterases would serve to maintain adequate levels of free CoA within the peroxisome by releasing CoA that is linked to non-metabolizable or poorly metabolized acyl-CoA intermediates generated by fatty acid oxidation. Thioesterases serving in this capacity would not only maintain adequate free CoA levels within the peroxisome, but would also facilitate the exit of the corresponding fatty acid from the peroxisome, since the free acid would then be capable of diffusion across lipid bilayers (26).

This model of thioesterase action may be extrapolated to metazoans, where multiple peroxisomal thioesterases could exist to handle a wider range of acyl CoA substrates. In mammals, peroxisomes participate in the metabolism of several fatty acids that cannot be degraded directly by the peroxisomal β-oxidation enzymes. Similar to the yeast model, mammalian peroxisomal thioesterases could release free CoA otherwise trapped on these compounds, thereby maintaining intraperoxisomal free CoA levels. However, it is also possible that mammalian peroxisomal thioesterases could play more directed roles in peroxisomal fatty acid

oxidation. For example, the α-oxidation of fatty acids begins with the conversion of acyl-CoAs to 2-hydroxy acyl-CoAs in a reaction catalyzed by PAHX (27–29). The 2-hydroxy acyl-CoAs produced could then be converted to 2-keto compounds by the action of peroxisomal oxidases. However, the three known human peroxiosomal 2-hydroxy acid oxidases (HAOXs) act on free fatty acids (21), and enzymes such as PTE1 and PTE2 may be required for the conversion of PAHX products to HAOX substrates. The 2-keto acids resulting from the successive action of PAHX, PTEs, and HAOXs could then be decarboxylated to regenerate free fatty acids. A model similar to this has been previously proposed by Wanders and colleagues to explain the breakdown of phytanic acid (22), a 3-methyl dietary fatty acid that must undergo one round of α-oxidation before it is degraded further by peroxisomal β-oxidation pathways. Further study is needed to determine if this model for thioesterase enzyme function is relevant *in vivo.*

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