Identification of Peroxisomal Acyl-CoA Thioesterases in Yeast and Humans*

(Received for publication, September 28, 1998, and in revised form, December 22, 1998)

Jacob M. Jones‡, Katja Nau§, Michael T. Geraghty¶, Ralf Erdmann§, and Stephen J. Gould‡

From the Departments of §Biological Chemistry and ¶Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 and the ¶Department of Physiological Chemistry, Ruhr-Universität Bochum, 44780 Bochum, Germany

A computer-based screen of the Saccharomyces cerevisiae genome identified YJR019C as a candidate oleate-induced gene. YJR019C mRNA levels were increased significantly during growth on fatty acids, suggesting that it may play a role in fatty acid metabolism. The YJR019C product is highly similar to tesB, a bacterial acyl-CoA thioesterase, and carries a tripeptide sequence, alanine-lysine-phenylalanineCOOH, that closely resembles the consensus sequence for type-I peroxisomal targeting signals. YJR019C directed green fluorescence protein to peroxisomes, and biochemical studies revealed that YJR019C is an abundant component of purified yeast peroxisomes. Disruption of the YJR019C gene caused a significant decrease in total cellular thioesterase activity, and recombinant YJR019C was found to exhibit intrinsic acyl-CoA thioesterase activity of 6 units/mg. YJR019C also shared significant sequence similarity with hTE, a human thioesterase that was previously reported here that hTE is also a peroxisomal protein, demonstrating that thioesterase activity is a conserved feature of peroxisomes. We propose that YJR019C and hTE be renamed as yeast and human PTE1 to reflect the fact that they encode peroxisomal thioesterases. The physical segregation of yeast and human PTE1 from the cytosolic fatty acid synthase suggests that these enzymes are unlikely to play a role in formation of fatty acids. Instead, the observation that PTE1 contributes to growth on fatty acids implicates this thioesterase in fatty acid oxidation.

Acyl-CoA thioesterases catalyze the hydrolysis of acyl-CoA molecules to free fatty acids and CoA. This enzymatic activity is an intrinsic component of animal fatty acid synthetase and in this context serves to terminate chain elongation (1). Additional thioesterases may associate noncovalently with fatty acid synthetase to modify the length of fatty acids produced by fatty acid synthetase in certain tissues. For instance, a mammalian gland-specific thioesterase has been shown to direct the production of medium chain fatty acids during lactation (2). Thioesterases of these types are the intranuclear thioesterases of peroxisomes and mitochondria. Although these fatty acid synthetase-associated thioesterases perform a relatively well understood role in cellular metabolism, eukaryotic cells contain a number of additional thioesterase activities with less well defined functions. The most notable of these are the intraorganellar thioesterases of peroxisomes and mitochondria.

Both peroxisomal and mitochondrial acyl-CoA thioesterases have been reported in mammalian cells (3–7). Peroxisomes and mitochondria do not contain fatty acid synthetase, and their thioesterases would not be expected to participate in the synthesis of fatty acids. In fact, the mere presence of thioesterases in these organelles seems counterproductive, because the oxidation of acyl-CoAs is a main function of both peroxisomes and mitochondria. Although the molecular cloning of mitochondrial fatty acyl-CoA thioesterase has been reported recently (5), the structural genes for peroxisomal acyl-CoA thioesterases remain to be identified. Here we report that YJR019C is a novel fatty acid-induced gene of the yeast Saccharomyces cerevisiae. This gene encodes a peroxisomal protein, and both genetic and biochemical evidence demonstrate that the YJR019C product is an acyl-CoA thioesterase. Loss of YJR019C interferes with the ability of yeast to grow on fatty acids, suggesting that this thioesterase plays an ancillary role in fatty acid oxidation rather than fatty acid synthesis. Furthermore, we report that this gene is conserved in humans and that the human form of this gene also encodes a peroxisomal thioesterase. The implications of these results for the role of peroxisomal thioesterases are discussed.

**EXPERIMENTAL PROCEDURES**

*Plasmids—The YJR019C open reading frame (ORF)1 was amplified from S. cerevisiae genomic DNA using the primers 5’-GGGAGATCTCAT-GAGGTCCTCCAAAATTGGCG-3’ and 5’-CCCGAGCTCAGGCCTCCTC-CCATTGCGAG-3’. The PCR product was digested with BglII and SacI (sites underlined) and cloned into the BglII and SacI sites of the yeast GFF fusion vector pGFP-X2 to make pGFP-YJR019C. The YJR019C ORF was reamplified by PCR from S. cerevisiae genomic DNA using the primers 5’-CCCGGATCCGGCTCGAGCATGTCGTCCGCTCCTGAGCACTATGGCCATG-3’ and 5’-CCCGGATCCTAGGCCTCCTCCTGAGCACTATGGCCATG-3’. This PCR product was digested with SacI and NotI (sites underlined) and cloned into the SacI and NotI sites of pMBP, a modified form of the pMALc2 expression vector (New England Biolabs) to make pJM19. The entire hTE ORF was amplified by PCR from human muscle cDNA using the primers 5’-CCCGGATCCTAGGCCTCCTCCTGAGCACTATGGCCATG-3’ and 5’-CCCGGATCCTAGGCCTCCTCCTGAGCACTATGGCCATG-3’. This PCR product was digested with SacI and NotI (sites underlined) and cloned into the SacI and NotI sites of pMBP, a modified form of the pMALc2 expression vector (New England Biolabs) to make pJM19.

The entire hTE ORF was amplified by PCR from human muscle cDNA using the primers 5’-CCCGGATCCTAGGCCTCCTCCTGAGCACTATGGCCATG-3’ and 5’-CCCGGATCCTAGGCCTCCTCCTGAGCACTATGGCCATG-3’. This PCR product was digested with SacI and NotI (sites underlined) and cloned into the SacI and NotI sites of pMBP, a modified form of the pMALc2 expression vector (New England Biolabs) to make pJM19.
5'-CCCTCTAGAGGGGGCGCCTGGTAATAGCTAGCTCTGTG-3'T

in conjunction with the first hTE primer above. These oligonucleotides append BamHI and XhoI sites to the 5' end and XhoI and NotI sites to the 3' end of the ORF. The PCR product was digested with BamHI and XhoI and cloned into the corresponding sites in pMAL2. The resulting plasmids were transformed into E. coli (20 μl Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol). The cell suspension was incubated on ice with 0.1 ml mg/ml lysozyme for 20 min. After this incubation the cells were frozen in liquid N2, thawed at 37 °C, and sonicated briefly at high intensity. This cycle of freezing, thawing, and sonication was repeated two times for a total of three cycles. A cleared lysate was generated by centrifugation at 17,500 × g for 20 min. Proteins were purified by one-step affinity chromatography using a 10-ml amylose-agarose (New England Biolabs) column according to the manufacturer’s instructions. Fractions were analyzed by SDS-PAGE, and those containing highly purified (>90% purity) MBP-YJR019C (Mw ~ 83,000) or MBP-hTEAKL (Mw ~ 70,000) were pooled and precipitated with 0.4 g/ml (NH4)2SO4. Aliquots of the purified fusion proteins were stored at −70 °C until needed.

**Whole Cell Lysates and Enzyme Assays**—For preparations of whole cell lysates, BY4733 cells or their yjr019C derivative were grown for 24 h in YPD, washed once with water, and transferred to YPOLT for an additional 24 h. Cells were incubated in reducing buffer (50 mM KPi, pH 7.4, 1 mM EDTA, and 10 mM 2-mercaptoethanol) for 20 min at 30 °C. At the end of the incubation period the lysate was centrifuged at 10,000 × g for 4 h for cells containing pMBP-hTE. ORF and pMBP-hTEAKL for the truncated form. The sequences of all ORFs in pMBP and pMAL2 were confirmed by automated fluorescent sequencing. The BamHI-XhoI fragment from pMBP-hTE was excised and transferred to the mammalian expression vector pcDNA3-Nmyc, a modified version of pcDNA3 (Invitrogen). This plasmid contains the sequence 5'-AAGCTTCCATCCAATGGCGGCGGTCCGGCTGGTGTCGATGGTGAGGTGACGACG3' between the HindIII and BamHI sites of pcDNA3 and is designed to express proteins in fusion with an amino-terminal myc epitope.

**Northern Blot Analysis**—BY4733 cells were maintained in midlog phase growth for 24–48 h in YPOLT or YPE medium. Two-liter cultures of cells were then harvested at an A600 of 1.0 and RNA was extracted using standard procedures (12). Poly(A)+ RNA was purified using Dynabeads according to the manufacturer’s directions (Dyna, Inc.). 0.5 μg of poly(A)+ RNA was loaded per lane, separated by denaturing agarose gel electrophoresis, and transferred to nylon membranes. Filters were prepared for hybridization and hybridized with radiolabeled DNA fragments using standard protocols (12) and washed with 0.1 M NaCl, 0.1% SDS at 58 °C for 4 h. The filters were exposed on X-ray film (Kodak). The HJR019C probe was from plasmid pGFP-YJR019C was used to transform the strain FY86 and its derivative to uracil auxotrophy. The yeast strain BY4733, SKQ2N (11), and BY4733, supplemented with 100 μM L-arginine was used to transform the strain FY86 and its derivative to uracil auxotrophy.

**Expression and Purification of Recombinant Proteins**—50-ml cultures of DH10B cells containing either the pMBP-hTEAKL or the pMYP-YJR019C plasmid were grown overnight at 37 °C in Luria broth supplemented with 100 μg/ml ampicillin. 40 ml of this culture were diluted into 1 liter of 2YT media (12) supplemented with 0.2% glucose and 100 μg/ml ampicillin. This culture was grown at 37 °C until the A600 reached 0.4, at which time isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM to induce protein expression. The induced culture was incubated at 37 °C for 4 h for cells containing pMBP-hTEAKL or 30 °C for 15 h for cells containing pMBP-YJR019C. Cells were harvested, washed with Luria broth, and resuspended in 25 ml of amylene (16). Cells were diluted into 1 liter of 2YT media supplemented with 100 μg/ml ampicillin and 10 mM 2-mercaptoethanol. The cell suspension was incubated on ice with 0.1 ml mg/ml lysozyme for 20 min. After this incubation the cells were frozen in liquid N2, thawed at 37 °C, and sonicated briefly at high intensity. This cycle of freezing, thawing, and sonication was repeated two times for a total of three cycles. A cleared lysate was generated by centrifugation at 17,500 × g for 20 min. Proteins were purified by one-step affinity chromatography using a 10-ml amylose-agarose (New England Biolabs) column according to the manufacturer’s instructions. Fractions were analyzed by SDS-PAGE, and those containing highly purified (>90% purity) MBP-YJR019C (Mw ~ 83,000) or MBP-hTEAKL (Mw ~ 70,000) were pooled and precipitated with 0.4 g/ml (NH4)2SO4. Aliquots of the purified fusion proteins were stored at −70 °C until needed.

**Transfections, Indirect Immunofluorescence, Antibodies, and Fluorescence Microscopy**—Indirect immunofluorescence studies were done with mouse monoclonal antibodies to PMP70 antibodies were obtained from Dave Valle and Gerardo Jimenez-Sanchez. The anti-myc mouse monoclonal antibody, anti-catalase (Catalase, 1:1000), and anti-PMP70 antibodies were obtained from Dave Valle and Gerardo Jimenez-Sanchez. The anti-myc mouse monoclonal antibody, anti-catalase sheep monoclonal antibody, and fluorescent secondary antibodies were obtained from commercial sources.

**Immunoblot and Subcellular Fractionation**—For immunoblot experiments, anti-hTE antibodies were affinity purified using MBP-hTEAKL or MBP-YJR019C. The enzyme was dissolved in buffer containing 0.1 M Tris-HCl, pH 7.5, 140 mM NaCl, and 0.2% Tween 20 and passed over the column. The column was washed with 30 bed volumes of TBST, and the purified anti-hTE antibodies were eluted with 100 mM glycine, pH 2.5, neutralized with 1/5 Tris-HCl, and

**Molecular Characterization of Peroxisomal Thioesterase**

**Growth Curves**—Preparation of high salt-extracted peroxisomal proteins from oleic acid-induced SKQ2N cells and their further separation by reverse phase HPLC was performed as described by Erdmann and Blobel (11). HPLC fractions were examined by SDS-PAGE. Fractions 26–28 were acid-induced SKQ2N cells and their further separation by reverse phase growth for 24–48 h in YPOLT or YPE medium. Two-liter cultures of cells were then harvested at an A600 of 1.0 and RNA was extracted using standard procedures (12). Poly(A)+ RNA was purified using Dynabeads according to the manufacturer’s directions (Dyna, Inc.). 0.5 μg of poly(A)+ RNA was loaded per lane, separated by denaturing agarose gel electrophoresis, and transferred to nylon membranes. Filters were prepared for hybridization and hybridized with radiolabeled DNA fragments using standard protocols (12) and washed with 0.1 M NaCl, 0.1% SDS at 58 °C for 4 h. The filters were exposed on X-ray film (Kodak). The HJR019C probe was from plasmid pGFP-YJR019C was used to transform the strain FY86 and its derivative to uracil auxotrophy. The yeast strain BY4733, SKQ2N (11), and BY4733, supplemented with 100 μM L-arginine was used to transform the strain FY86 and its derivative to uracil auxotrophy.
precipitated with 0.4 g/ml (NH₄)₂SO₄ for storage. Anti-myc polyclonal antibodies were obtained from Santa Cruz Biotechnology. Expression of proteins in rabbit reticulocyte lysates (Promega) was according to the manufacturer's instructions, using the pcDNA3-Nmyc plasmid containing either the hTE open reading frame or that of an unrelated peroxisomal enzyme. Subcellular fractionation of rat liver was as described by Mihalik (16). Immunoblots were performed as described by Crane et al. (17).

RESULTS

YJR019C Is an Oleate-induced Gene—A screen for potential fatty acid-regulated genes in yeast identified a consensus oleate response element (CGGN₃TNAN(6–13)(C/G)CG) in the YJR019C promoter region (Fig. 1).² To test whether YJR019C is regulated by fatty acids, we determined the relative abundance of YJR019C mRNA in yeast grown with 0.2% oleate or 0.2% glucose as the sole carbon source. As shown in Fig. 2, oleate stimulated YJR019C expression approximately sixfold, whereas glucose had no effect. This suggests that YJR019C is an oleate-responsive gene.

Fig. 1. Nucleotide and predicted protein sequence of the YJR019C gene. The open reading frame is shown with 500 bp of flanking sequence at both the 5' and 3' ends. A consensus oleate response element (underlined) is present from nucleotides 2130 to 2108 relative to the first nucleotide of the YJR019C open reading frame. The 1050-bp open reading frame, which terminates in the atypical type 1 peroxisomal-targeting sequence alanine-lysine-phenylalanineCOOH (underlined), is predicted to encode a basic protein (pI, 8.82) with a mass of 40.2 kDa.

Molecular Characterization of Peroxisomal Thioesterase

V V Y G S E R I N A K E

precipitated with 0.4 g/ml (NH₄)₂SO₄ for storage. Anti-myc polyclonal antibodies were obtained from Santa Cruz Biotechnology. Expression of proteins in rabbit reticulocyte lysates (Promega) was according to the manufacturer's instructions, using the pcDNA3-Nmyc plasmid containing either the hTE open reading frame or that of an unrelated peroxisomal enzyme. Subcellular fractionation of rat liver was as described by Mihalik (16). Immunoblots were performed as described by Crane et al. (17).
dance of YJR019C mRNA in yeast grown in medium containing either oleic acid or ethanol. Both oleic acid and ethanol are converted to acetyl-CoA before use in further metabolic or biosynthetic pathways. Therefore, any increases in mRNA levels in yeast grown on oleic acid versus those grown on ethanol should reflect changes specific to fatty acid metabolism. Polyadenylated RNA was extracted from log phase yeast grown in either oleic acid medium (YPOLT) or ethanol medium (YPE) and analyzed by Northern blot with the YJR019C gene as probe (Fig. 2). Although a significant amount of YJR019C mRNA was present in ethanol-grown cells, the level of YJR019C mRNA was increased greatly in oleate-grown cells. The promoter region of YJR019C lacks ORE-like elements, and its mRNA was present at similar levels in cells grown in either oleic acid or ethanol medium.

The YJR019C Gene Encodes a Peroxisomal Protein—Growth of yeast on fatty acids requires a wide array of peroxisomal proteins. Because YJR019C was induced by fatty acids and encodes a protein with a PTS1-like sequence at its carboxyl terminus (alanine-lysine-phenylalanine COOH), we tested whether the YJR019C gene product might be located in peroxisomes. Previous studies have established that GFP can be targeted to peroxisomes by fusion to peroxisomal proteins (18). We therefore fused the GFP ORF to the amino-terminal end of the YJR019C ORF in an oleate-inducible expression vector and introduced this vector into the wild-type yeast strain FY86. The distribution of the GFP-YJR019C fusion protein was assessed by confocal and phase contrast microscopy (Fig. 3A). GFP fluorescence was present in discrete punctate structures typical of S. cerevisiae peroxisomes. To determine whether these structures were in fact peroxisomes, we next examined the distribution of this fusion protein in a pex3Δ derivative of FY86 (PEX3 is required for peroxisomal matrix protein import; Ref. 19). In the pex3 strain, GFP fluorescence was found throughout the cytoplasm (Fig. 3B), a distribution that is expected for a peroxisomal matrix protein.

The identification of YJR019C as a peroxisomal protein was also accomplished by a direct biochemical approach. In an independent attempt to identify the constituents of peroxisomes, highly purified peroxisomes were isolated from yeast grown in oleic acid medium. Soluble matrix proteins were removed by hypotonic lysis of the peroxisomes, and membrane-associated proteins were released by high salt wash of the peroxisomal membrane. The proteins released by high salt extraction were then separated by reverse phase HPLC as described previously (11). HPLC fractions were separated further by SDS-PAGE, transferred to a membrane, and visualized by Amido Black staining. Individual bands were then subjected to amino-terminal sequence analysis. The amino-terminal sequence of an ~40-kDa protein, which eluted in fractions 26–28, corresponded to the deduced amino terminus of YJR019C (Fig. 4). These results demonstrated that YJR019C is an abundant membrane-associated component of yeast peroxisomes.

YJR019C Encodes an Acyl-CoA Thioesterase and Contributes to Growth on Fatty Acids—Because YJR019C mRNA was highly induced by oleic acid and encoded a peroxisomal protein, we next tested whether YJR019C might have a role in growth on fatty acids. A yjr019cΔ derivative of BY4733 was created, and its growth on oleic acid medium was compared with that of the wild-type BY4733 strain and a pex8 derivative of BY4733. The yjr019cΔ cells exhibited a partial growth defect, growing to 53% of the final wild-type density, whereas the pex8 strain showed the typical pex phenotype, growing to ~20% of wild-type density (Fig. 5A). Thus, YJR019C appears to play an ancillary role in growth on fatty acids.

The deduced product of YJR019C shared significant amino acid similarity with tesB, a bacterial acyl-CoA thioesterase (20). Disruption of YJR019C resulted in a loss of ~80% of the total n-decanoyl-CoA esterase activity in yeast cells (Fig. 5B). To test directly whether YJR019C is an acyl-CoA thioesterase, we expressed and purified YJR019C in fusion with maltose-binding protein. MBP-YJR019C had significant acyl-CoA thioesterase activity using n-decanoyl-CoA as substrate, exhibiting a specific activity of 6.1 units/mg at 30 °C.

The Human Acyl-CoA Thioesterase hTE Is Homologous to YJR019C—The amino acid sequence of YJR019C was used to scan the data base of expressed sequence tags for any human
Molecular Characterization of Peroxisomal Thioesterase

Fig. 4. Preparative chromatographic separation of high salt extract of peroxisome membranes for microsequence analysis. Highly purified peroxisomes were disrupted by hypotonic lysis, and soluble matrix proteins were removed by low salt extraction. Membrane-associated proteins were released by high salt extraction and separated by reverse phase HPLC. Fractions 26–47 were further separated by SDS-PAGE and visualized by Coomassie Blue staining. The position of the YJR019C gene product is indicated by an arrowhead. The amount per lane corresponds to 5% of the total fraction. Molecular mass standards are indicated on the left.

Fig. 5. YJR019C is an acyl-CoA thioesterase involved in fatty acid metabolism. A, the growth of wild-type (open circle), yjr019cΔ (filled circle), and pex8Δ (open triangle) cells on oleic acid was monitored spectrophotometrically over 168 h. The yjr019cΔ cells grew to 53% of the final wild-type density. B, lysates were prepared from oleate-grown wild-type (WT) and yjr019cΔ cells. Equal amounts of protein from each lysate were assayed for acyl-CoA thioesterase activity. The lysate of the wild-type strain had a specific activity of 1.8 × 10^2 unit/mg, whereas the lysate of yjr019cΔ strain had a specific activity of 4.0 × 10^3 unit/mg.

Homologues of this yeast thioesterase. Multiple human expressed sequence tags were identified, all of which corresponded to a single, previously characterized gene, hTE (21, 22). The deduced amino acid sequence of hTE shows 26.3% identity to YJR019C and, like its yeast counterpart, contains a PTS1 motif (serine-lysine-leucineCOOH) at its carboxyl terminus (Fig. 6). Although hTE has been shown previously to have acyl-CoA thioesterase activity, we tested its activity as an MBP fusion for direct comparison with YJR019C. MBP-hTE displayed a specific activity of 12 units/mg hTE at 37 °C, an activity similar to that of MBP-YJR019C.

The hTE Gene Encodes a Peroxisomal Protein—Given that the hTE gene product is similar to YJR019C and has a canonical PTS1 sequence, we tested whether hTE encoded a peroxisomal protein. To address this issue, we first modified the hTE ORF to include a 10-amino acid myc tag at its 5' end. A plasmid designed to express this myc-hTE fusion, pcDNA3-Nmyc-hTE, was introduced into human skin fibroblasts. Indirect immunofluorescence experiments revealed that Nmyc-hTE was localized to peroxisomes (Fig. 7A), as determined by colocalization with the peroxisomal marker protein PMP70 (Fig. 7B). To assess whether Nmyc-hTE was imported into the peroxisome lumen, we repeated these experiments under differential permeabilization conditions. Skin fibroblasts expressing pcDNA3-Nmyc-hTE were permeabilized with a limiting amount of digitonin, which permeabilizes the plasma membrane but does not permeabilize the peroxisomal membrane. Indirect immunofluorescent labeling of Nmyc-hTE in digitonin-permeabilized cells showed only background cytosolic staining, even though PMP70-containing peroxisomes were readily detected (Fig. 7, C and D). The PMP70 antibodies that were used in these experiments recognize the cytosolic domain of this peroxisomal membrane protein. These results demonstrate that Nmyc-hTE is translocated into the peroxisome lumen.

Zellweger syndrome is caused by defects in genes that are required for peroxisome biogenesis, and cells from Zellweger syndrome patients display defects in peroxisomal matrix protein import (23). As an independent test of whether hTE is a peroxisomal protein, we expressed Nmyc-hTE in the Zellweger syndrome cell line PBD100. This 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 (14). Nmyc-hTE accumulated in the cytosol of PBD100 cells, as would be expected for a peroxisomal matrix protein, although intact peroxisomes were detected using antibodies to PMP70 (Fig. 7, E and F).

Previous studies identified hTE as a protein that interacts with HIV-Nef (21, 22). Given that there is no evidence that HIV-Nef is peroxisomal, we tested whether endogenously synthesized hTE was located in peroxisomes using antibodies raised against a bacterially synthesized version of hTE. The immune sera recognize peroxisomes, as evident from their colocalization with the peroxisomal marker protein catalase (Fig. 8, A and B). In contrast, the preimmune sera do not recognize peroxisomes (Fig. 8, C and D).

The distribution of this mammalian thioesterase was also assessed by subcellular fractionation experiments. We first
generated affinity-purified anti-hTE antibodies and tested whether they recognized the product of the hTE gene. Rabbit reticulocyte lysates were used to synthesize either Nmyc-hTE or an unrelated myc-tagged protein, and the two lysates were then examined by immunoblot. Affinity purified anti-hTE antibodies recognized a protein of the correct molecular mass only in the lysate in which Nmyc-hTE was synthesized (Fig. 9A).

Anti-myc polyclonal antibodies confirmed expression of both proteins (data not shown). Next, a postnuclear supernatant was prepared from rat liver and separated by Nycodenz density gradient centrifugation. Fractions across the gradient were examined for the presence of hTE by immunoblot using anti-myc antibodies. In agreement with previous studies, hTE was found in the peroxisomal fraction (Fig. 9B).

Next, a postnuclear supernatant was prepared from rat liver and separated by Nycodenz density gradient centrifugation. Fractions across the gradient were examined for the presence of hTE by immunoblot using anti-myc antibodies. In agreement with previous studies, hTE was found in the peroxisomal fraction (Fig. 9B).

FIG. 6. Amino acid alignment of YJR019C and hTE proteins. Sequence alignment was performed using DNASTAR (Madison, WI) and the PAM 250 matrix. Identical residues are boxed.

FIG. 7. hTE is a peroxisomal matrix protein. Human skin fibroblasts expressing Nmyc-hTE were processed for double indirect immunofluorescence by fixing cells and permeabilizing with 1% Triton X-100. The distribution of Nmyc-hTE was examined using anti-myc (A) and anti-PMP70 (B) antibodies. Additional cells from the same set expressing Nmyc-hTE were permeabilized with 25 μg/ml digitonin and examined again using anti-myc (C) and anti-PMP70 (D) antibodies. The distribution of Nmyc-hTE was also examined in the PEX10-deficient cell line PBD100, again by double indirect immunofluorescence using anti-myc (E) and anti-PMP70 (F) antibodies. Scale bar, 25 μm.

FIG. 8. Endogenously synthesized hTE colocalizes with the peroxisomal matrix enzyme catalase. HepG2 cells were processed for double indirect immunofluorescence using anti-hTE antiserum (A) or preimmune serum (C) and anti-catalase (B and D) antibodies. Scale bar, 25 μm.
Molecular Characterization of Peroxisomal Thioesterase

Fig. 9. Anti-hTE antibodies recognize a 36-kDa protein in peroxisomal and cytoplasmic subcellular fractions. A, rabbit reticuloocyte lysate was used to synthesize Nyc-hTE (lane 1) or an unrelated peroxisomal protein (lane 2). Equal amounts of each lysate were separated by SDS-PAGE and analyzed by immunoblot with affinity-purified anti-hTE antibodies. B, postnuclear supernatant from rat liver was fractionated by Nycodenz density centrifugation. Equal amounts of fractions were assayed for catalase (dark bars) and succinate dehydrogenase (light bars) activity. Equal amounts of fractions were also analyzed by immunoblot using affinity-purified anti-hTE antibodies. The bar graph shows the relative amounts of the peroxisomal and mitochondrial marker enzyme activities in each fraction. The lower panels show the distribution of the ~36 kDa rat protein recognized by the anti-hTE antibodies.

assayed for peroxisomal and mitochondrial markers as well as by immunoblot with the affinity purified anti-hTE antibodies (Fig. 9B). These antibodies detected a 36-kDa protein in rat liver peroxisomes, as well as in cytosolic fractions at the top of the gradient. Peroxisomes will rupture during homogenization and centrifugation, releasing peroxisomal matrix proteins to the cytosol. Peroxisome rupture in this experiment is confirmed by the presence of significant catalase activity in the fractions at the top of the gradient. Although the amount of cytoplasmic thioesterase may be partly explained by peroxisome rupture, we cannot rule out the possibility that a small pool of thioesterase exists in the cytoplasm at steady state. These results support the hypothesis that hTE is a peroxisomal protein but do not rule out the possibility that some hTE may be cytosolic and available to interact with HIV-Nef.

DISCUSSION

In this report we have demonstrated that YJR019C is an oleate-regulated gene that encodes a novel peroxisomal protein. Furthermore, we find that YJR019C is required for the majority of acyl-CoA thioesterase activity in yeast cells and show that recombinant YJR019C displays significant acyl-CoA thioesterase activity in vitro. The physical segregation of peroxisomal YJR019C from cytosolic fatty acid synthetase, the enzyme required for fatty acid synthesis, provides compelling evidence that the peroxisomal thioesterase is not involved in fatty acid synthesis. Our finding that loss of YJR019C results in impaired growth on fatty acids implicates this gene in fatty acid oxidation, a process that is exclusively peroxisomal in yeast. The fact that the human homologue of YJR019C, the previously identified hTE gene, also encodes a peroxisomal thioesterase suggests that there may be a conserved role for this enzyme in peroxisomal fatty acid oxidation. We propose that YJR019C and human hTE both be renamed PTE1 to reflect the peroxisomal distribution and acyl-CoA thioesterase activity of their gene products.

Fatty acids must be esterified with CoA before their β oxidation. Thus, it is somewhat paradoxical that a peroxisomal acyl-CoA thioesterase is required for normal growth of yeast on fatty acids. In fact, our current understanding of peroxisomal fatty acid oxidation in yeast provides no direct role for thioesterase activity in fatty acid β oxidation or growth on fatty acids. Therefore, it is important to consider possible indirect roles for PTE1 in fatty acid oxidation. Previous studies have established that the peroxisome membrane is impermeable to small molecules such as NAD and NADP (24, 25). As a result, loss of enzymes involved in NAD regeneration lead to loss of NAD-dependent activities within the peroxisome, including fatty acid oxidation. If the peroxisome membrane is also impermeable to CoASH, then the intraperoxisomal free CoASH pool may also be dependent on constant regeneration. Previous studies in yeast have established that CoASH regeneration would occur via complete oxidation of acyl-CoA to acetyl-CoA, followed by transfer of acetate to carnitine and release of CoASH in a reaction catalyzed by CAT1 (26). However, if CoA were appended to poorly metabolized or nonmetabolizable fatty acids, CoASH levels would be expected to fall as more and more CoA was incorporated into these metabolic sinks. In this scenario, a peroxisomal thioesterase that acted primarily at high acyl-CoA and/or CoASH levels might serve a stimulatory role in fatty acid oxidation by providing an alternative mechanism for generating the free CoASH that is necessary for fatty acid β oxidation. As for the fate of the fatty acids that may be released, these would be free to equilibrate with cellular and extracellular pools, reducing their concentration in the peroxisome.

This indirect model for PTE function has the potential to explain the fact that loss of PTE1 impairs growth of yeast on fatty acids, but we readily admit that there is not yet any independent corroborating evidence in its favor. As such, it should be viewed with skepticism. At the same time, it is worthwhile to consider this model as a possible explanation for the role of mammalian PTE1. If CoASH depletion does serve to inhibit peroxisomal fatty acid β oxidation in yeast, we might also expect CoASH depletion to pose problems for fatty acid oxidation in mammalian peroxisomes and mitochondria. In fact, a similar hypothesis has been proposed to explain the function of rat mitochondrial thioesterase (6).

An alternative role for mammalian peroxisomal thioesterases has also been suggested (7). Peroxisomal β oxidation in mammalian cells differs from the yeast system in that it does not oxidize fatty acids completely. Instead, it transfers medium chain fatty acids out of the peroxisome as acyl-carnitine (27). These are then exported to mitochondria where their oxidation is completed. This facet of mammalian peroxisomal fatty acid oxidation led to the hypothesis that peroxisomal thioesterases may serve to regulate the chain length at which fatty acids are exported (7). However, there are three flaws with this model. First, mammalian peroxisomal carnitine acyltransferase accepts fatty acids in the form of CoA esters, not as free acids (28). Thus, it is unclear how a thioesterase would contribute to acyl-carnitine export. Second, the chain length specificities of mammalian peroxisomal β-oxidation enzymes are thought to be sufficient to control the extent to which fatty acyl-CoAs are shortened in peroxisomes (7, 27). Third, this model cannot explain the presence of a thioesterase in yeast peroxisomes, because they oxidize fatty acids to completion (29).

The identification of PTE1 in both yeast and human cells...
raises the question of whether they correspond to previously described peroxisomal enzymes. This is clearly not the case for yeast PTE1, because this report is the first description of this enzyme in yeast peroxisomes. However, there have been several reports of peroxisomal thioesterases in mammalian cells (4, 7). A purification of rat liver peroxisomal thioesterases revealed that myristoyl-CoA thioesterase activity eluted in two peaks, one of ~35 kDa and one of larger size (4). The product of human PTE1 has a deduced Mr of 36 kDa and may represent the human homologue of the smaller rat peroxisomal thioesterase. A second study found peroxisomal thioesterase activity to be present in a broad peak of ~46 kDa (7). However, subsequent fractionation of this sample led to the purification of a single thioesterase of ~46 kDa. Interestingly, the putative cytosolic thioesterase 1 contains a PTS1-like sequence at its carboxyl terminus, proline-lysine-isoleucineCOOH, and has a deduced Mr of 46 kDa (30). A further analysis of cytosolic thioesterase 1 distribution may resolve the question of whether it actually represents a second, 46-kDa thioesterase of peroxisomes.

The localization of human PTE1 (hTE) to the peroxisome lumen was observed for an overexpressed, epitope-tagged version of HsPTE1 as well as for the endogenously synthesized, wild-type HsPTE1 of human fibroblasts. Thus, there is little doubt that this protein is predominantly peroxisomal. However, it is also true that human PTE1 (hTE) was first identified because of its interaction with HIV-Nef in the yeast two-hybrid system (21, 22). There is no indication that Nef is associated with peroxisomes, nor is there any known role for peroxisomes in the life cycle of HIV. Nevertheless, the interaction between PTE1 (hTE) and HIV-Nef was also detected in lysates of CEM cells that stably expressed HIV-Nef. Although PTE1-Nef interaction might have occurred after the formation of the cell-free lysate in these experiments, it is also quite possible that small amounts of PTE1 are present in the cytosol. Our fractionation data cannot rule out the possibility that some PTE1 may be cytoplasmic at steady state and available for interaction with HIV-Nef. Also, it is possible that HIV-Nef may bind PTE1 before its import into peroxisomes.

Acknowledgments—We thank Jianwu Bai and James Morrell for technical assistance and Brian Geisbrecht, Katie Sacksteder, and Stephanie Mihalik for assistance with subcellular fractionation experiments.

REFERENCES
1. Smith, S. (1994) FASEB J. 8, 1248–1259
2. Liberti, L. J., and Smith, S. (1978) J. Biol. Chem. 253, 1393–1401
3. Svensson, L. T., Alexson, S. E. H., and Hultumen, J. K. (1995) J. Biol. Chem. 270, 12177–12183
4. Svensson, L. T., Wilcke, M., and Alexson, S. E. H. (1995) Eur. J. Biochem. 230, 813–820
5. Svensson, L. T., Engberg, S. T., Toshifumi, A., Usada, N., Alexson, S. E. H., and Hashimoto, T. (1998) Biochem. J. 329, 601–608
6. Lee, K. Y., and Schulz, H. (1979) J. Biol. Chem. 254, 4516–4523
7. Wilcke, M., and Alexson, S. E. H. (1984) Eur. J. Biochem. 122, 803–811
8. Grant, S. G., Jessee, J., Bloom, R. F., and Hanahan, D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4645–4649
9. Winston, F., Boeke, J. D., and Dancis, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 13280–13284
10. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
11. Fernandez, J., Demott, M., Atherton, D., and Mische, S. M. (1992) Anal. Biochem. 201, 569–573
12. Warren, D. S., Morrell, J. C., Moser, H. W., Valle, D., and Gould, S. J. (1998) Am. J. Hum. Genet. 63, 347–359
13. Slawekii, M., Dott, G., Steinberg, S., Moser, A. B., Moser, H. W., and Gould, S. J. (1995) J. Cell Sci. 108, 1817–1829
14. Mihalik, S. J. (1992) Prog. Clin. Biol. Res. 357, 239–244
15. Crane, D. I., Kalish, J. E., and Gould, S. J. (1994) J. Biol. Chem. 269, 21835–21844
16. Kalish, J. E., Keller, G. A., Morrell, J. C., Mihalik, S. J., Smith, B., Cregg, J. M., and Gould, S. J. (1996) EMBO J. 15, 3275–3285
17. Holzbaur, J., Veinhaus, M., and Kunau, W. H. (1991) J. Cell Biol. 114, 1167–1178
18. Naggett, J., Narasimhan, M. L., DeVeaux, L., Cho, H., Randhawa, Z. I., Cronan, J. E., Jr., Green, B. N., and Smith, S. (1991) J. Biol. Chem. 266, 11044–11050
19. Liu, L. X., Margottin, F., Le Gall, S., Schwartz, O., Selig, L., Benarous, R., and Benichou, S. (1997) J. Biol. Chem. 272, 13773–13785
20. Watanabe, H., Shiratori, T., Itoh, H., Miyatake, S., Okaizuki, Y., Ikuta, K., Sato, T., and Saito, T. (1997) Biochem. Biophys. Res. Commun. 238, 234–239
21. Lazarow, P. B., and Moser, H. W. (1995) in The Metabolic and Molecular Bases of Inherited Disease (Scriver, C., Beaudet, A., Sly, W., and Valle, D., eds), 7th Ed., pp. 2287–2324, McGraw-Hill, New York
22. van der Romou, C. W., Hettema, E. H., Kal, A. J., van den Berg, M., Tabak, H. F., and Wanders, R. J. (1998) EMBO J. 17, 677–687
23. van der Romou, C. W., Eldersma, Y., Singh, N., Wanders, R. J., and Tabak, H. F. (1995) EMBO J. 14, 3480–3486
24. Eldersma, Y., van der Romou, C. W., Wanders, R. J., and Tabak, H. F. (1995) EMBO J. 14, 3472–3479
25. Mannenerts, G., and van Veldhoven, P. (1999) Ann. NY Acad. Sci. 894, 99–115
26. Farrell, S. O., Fiol, C. J., Reddy, J. K., and Bieber, L. L. (1984) J. Biol. Chem. 259, 13089–13095
27. Kunau, W.-H. (1992) FEBS Lett. 308, 1–6
28. Warren, D. S., Morrell, J. C., Moser, H. W., Valle, D., and Gould, S. J. (1998) EMBO J. 15, 3275–3285
29. Liu, L. X., Margottin, F., Le Gall, S., Schwartz, O., Selig, L., Benarous, R., and Benichou, S. (1997) J. Biol. Chem. 272, 13773–13785
30. Watanabe, H., Shiratori, T., Itoh, H., Miyatake, S., Okaizuki, Y., Ikuta, K., Sato, T., and Saito, T. (1997) Biochem. Biophys. Res. Commun. 238, 234–239
31. Lazarow, P. B., and Moser, H. W. (1995) in The Metabolic and Molecular Bases of Inherited Disease (Scriver, C., Beaudet, A., Sly, W., and Valle, D., eds), 7th Ed., pp. 2287–2324, McGraw-Hill, New York
32. van der Romou, C. W., Hettema, E. H., Kal, A. J., van den Berg, M., Tabak, H. F., and Wanders, R. J. (1998) EMBO J. 17, 677–687
33. van der Romou, C. W., Eldersma, Y., Singh, N., Wanders, R. J., and Tabak, H. F. (1995) EMBO J. 14, 3480–3486
34. Eldersma, Y., van der Romou, C. W., Wanders, R. J., and Tabak, H. F. (1995) EMBO J. 14, 3472–3479
35. Mannenerts, G., and van Veldhoven, P. (1999) Ann. NY Acad. Sci. 894, 99–115
36. Farrell, S. O., Fiol, C. J., Reddy, J. K., and Bieber, L. L. (1984) J. Biol. Chem. 259, 13089–13095
37. Kunau, W.-H. (1992) Prog. Clin. Biol. Res. 375, 9–18
38. Lindquist, P. J., Svensson, L. T., and Alexson, S. E. H. (1998) Eur. J. Biochem. 251, 631–640