Regulation of Peroxisome Size and Number by Fatty Acid β-Oxidation in the Yeast Yarrowia lipolytica*

Received for publication, November 19, 1999, and in revised form, April 25, 2000
Published, JBC Papers in Press, April 27, 2000, DOI 10.1074/jbc.M909285199

Jennifer J. Smith†, Trevor W. Brown, Gary A. Eitzen‡, and Richard A. Rachubinski¶

From the Department of Cell Biology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

The Yarrowia lipolytica MFE2 gene encodes peroxisomal β-oxidation multifunctional enzyme type 2 (MFE2). MFE2 is peroxisomal in a wild-type strain but is cytosolic in a strain lacking the peroxisomal targeting signal (PTS1) receptor. MFE2 has a PTS1, Ala-Lys-Leu, that is essential for targeting to peroxisomes. MFE2 lacking a PTS1 can apparently oligomerize with full-length MFE2 to enable targeting to peroxisomes. Peroxisomes of an oleic acid-induced MFE2 deletion strain, mfe2-KO, are larger and more abundant than those of the wild-type strain. Under growth conditions not requiring peroxisomes, peroxisomes of mfe2-KO are larger but less abundant than those of the wild-type strain, suggesting a role for MFE2 in the regulation of peroxisome size and number. A nonfunctional version of MFE2 did not restore normal peroxisome morphology to mfe2-KO cells, indicating that their phenotype is not due to the absence of MFE2. mfe2-KO cells contain higher amounts of β-oxidation enzymes than do wild-type cells. We also show that increasing the level of the β-oxidation enzyme thiolase results in enlarged peroxisomes. Our results implicate peroxisomal β-oxidation in the control of peroxisome size and number in yeast.

Cellular fatty acid degradation into acetyl-CoA units occurs by β-oxidation, a cyclic reaction that shortens fatty acids by two carbon atoms per round. In higher eukaryotes, β-oxidation systems are found in both mitochondria and peroxisomes (reviewed in Ref. 1), whereas in lower eukaryotes such as yeast, this process occurs exclusively in peroxisomes (reviewed in Ref. 2). The enzymes that contribute to β-oxidation in yeast include, in order, acyl-CoA oxidase, multifunctional enzyme type 2 (MFE2)† (with 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities) (3, 4), and the cleavage enzyme, 3-ketoacyl-CoA thiolase.

Peroxisomes are proteins that function in peroxisome assembly. Enzymes involved in peroxisomal β-oxidation are synthesized on free polysomes and then imported into the matrix of the peroxisome in an evolutionarily conserved manner that is dependent on a subset of peroxins and on cis-acting peroxisomal targeting signals (PTSs) (reviewed in Refs. 5 and 6). Proteins containing a PTS1 (a carboxyl-terminal tripeptide of the sequence SKL or a conserved variant thereof) require several peroxins for their import, including Pex5p, which binds directly to PTS1s. Proteins containing a PTS2, which has the consensus sequence (R/K)(L/V/I)X(Q/H)/LA and is found at or near the amino termini of a small subset of peroxisomal matrix proteins, also require several peroxins for their import, including Pext7p, which binds directly to PTS2s. An interesting feature of peroxisomes is their ability to import oligomeric proteins (7, 8). 3-ketoacyl-CoA thiolase, the enzyme catalyzing the last step of peroxisomal β-oxidation, has been shown to be imported as a homodimer (7, 9).

In many cell types, peroxisomes increase in size and number in response to certain extracellular stimuli (reviewed in Ref. 10). Evidence suggests that this regulation is controlled by a subset of peroxins. In yeast, Pex10p (11), Pex11p (12–15), and Pex16p (16) have each been shown to play a role in regulating the size and/or number of peroxisomes in response to growth on carbon sources metabolized by peroxisomes. In mammalian cells, PEX11β has been shown to induce peroxisome proliferation in the absence of an external stimulus (e.g. a hypolipidemic drug such as clofibrate) (17). Other evidence suggests that there is also metabolic control of peroxisome abundance and size in mammalian cells. Cells of a patient with a specific deficiency in acyl-CoA oxidase were shown to have enlarged peroxisomes that are heterogeneous in size (18). It has also been reported that human cells lacking either acyl-CoA oxidase or MFE2 have a 5-fold reduction in peroxisome abundance and a 2–4-fold increase in peroxisome diameter, as compared with normal cells (19).

Here, we report the identification and characterization of the MFE2 gene of the yeast Yarrowia lipolytica and the study of the targeting of its encoded protein, MFE2, to peroxisomes. We also provide evidence of a role for β-oxidation enzymes in the regulation of peroxisome size and number in Y. lipolytica, the first evidence for this type of metabolic control in yeast.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—The yeast strains used in this study are listed in Table I. Media components were as follows: YEPD, 1% yeast extract, 2% peptone, 2% glucose; YEP, 1% yeast extract, 2% peptone, 2% sodium acetate; YPBO, 0.3% yeast extract, 0.5% peptone, 0.5% K2HPO4, 0.5% KH2PO4, 1% Brij 35, 1% (w/v) oleic acid, YND, 1.34% yeast nitrogen base without amino acids, 2% glucose; YNO, 1.34% yeast nitrogen base without amino acids, 0.05% (w/v) Tween 40, 0.2% (w/v) oleic acid; YNA, 1.34% yeast nitrogen base without amino acids.
Isolation and Sequencing of the MFE2 Gene—A fragment of the MFE2 gene was isolated serendipitously from the DNA of a Y. lipolytica strain containing a PEX16 disruption construct (16) spuriously integrated into its genome. This strain was unable to use oleic acid as a carbon source. A fragment containing the disruption construct and flanking genomic DNA was recovered from this strain and ligated into pGEM7Zf+(Promega) to generate the plasmid p6AD. By sequencing, a 1146-bp fragment of the MFE2 gene (nucleotides 1090–2235, where +1 is the "A" nucleotide of the initiation codon) was identified within the disruption construct. To clone the entire MFE2 gene, Y. lipolytica genomic DNA was isolated from the wild-type strain E122, digested with EcoRI, and fractionated by size. By polymerase chain reaction (PCR) amplification of DNA fragments within each fraction using oligonucleotides 731 and 742 (Table II), it was determined that fragments of DNA containing the MFE2 gene were between 6 and 8 kilobase pairs in size. EcoRI-digested genomic DNA of this size range was ligated into pGEM7Zf(+) to construct a library for colony hybridization. A fragment of the MFE2 gene amplified by PCR from p6AD using oligonucleotides 731 and 742 was radiolabeled with [α-32P]dATP and used to probe this library. One plasmid, pG7MFE2, was isolated from a positive colony, and the fragment containing the open reading frame (ORF) of the MFE2 gene was used in place of the MFE2 gene in the expression plasmid, pG7MFE2 was digested with SphI, and the fragment containing the open reading frame was ligated into the unique SphI site of pGEM-7Zf(-) to generate the plasmid pMyc-MFE2 encoding Myc-MFE2. This plasmid was digested with SphI, and the fragment containing the modified MFE2 gene, as well as 1066 and 426 bp of sequence upstream and downstream of the ORF, respectively, was ligated into the Escherichia coli Y. lipolytica shuttle vector pINA445 (20) cut with SphI to make the plasmid pMyc-MFE2 encoding MFE2. pINA445 contains the Y. lipolytica LEU2 gene for the positive selection of yeast transformants and the Y. lipolytica ARS68 gene for autonomous plasmid replication in Y. lipolytica cells.

Integrative Disruption of the MFE2 and PEX5 Genes—The flanking regions of the MFE2 ORF were amplified from pG7MFE2 using oligonucleotides 922 and 945 and ligated into the unique SphI site of pGEM7Zf(+) to construct a library for colony hybridization. A fragment of the MFE2 gene amplified by PCR from p6AD using oligonucleotides 731 and 742 was radiolabeled with [α-32P]dATP and used to probe this library. One plasmid, pG7MFE2, was isolated from a positive colony, and both strands of the ~6.8-kilobase pairs insert were sequenced. The sequence of the MFE2 protein was deduced from the gene nucleotide sequence and compared with protein data bases using the BLAST algorithm provided by the network service of the National Center for Biotechnology Information (Bethesda, MD).

To make an MFE2 expression plasmid, pG7MFE2 was digested with SphI, and the fragment containing the open reading frame (ORF) of the MFE2 gene, as well as 1066 and 426 bp of sequence upstream and downstream of the ORF, respectively, was ligated into the Escherichia coli Y. lipolytica shuttle vector pINA445 (20) cut with SphI to make the plasmid pMyc-MFE2 encoding MFE2. pINA445 contains the Y. lipolytica LEU2 gene for the positive selection of yeast transformants and the Y. lipolytica ARS68 gene for autonomous plasmid replication in Y. lipolytica cells.

Restriction endonuclease recognition sites are underlined. Mutations introduced by site-directed mutagenesis are indicated in boldface.
were fixed in 3.7% formaldehyde for 2 or 30 min as indicated and processed for immunofluorescence microscopy as described (23), except that spheroplast preparation was done in 100 mM potassium phosphate (pH 7.5), 1.2% sorbitol, 40 μg of Zymolyase-100T (ICN, Aurora, OH)/ml, 28 mM 2-mercaptoethanol for 30 min at 30 °C with gentle agitation. Imaging was done using a Spot Cam digital fluorescence camera (Spot Diagnostic Instruments, Sterling Heights, MI).

**Electron Microscopy**—Whole cells were fixed in 1.5% K2O4O2 for 20 min at room temperature, dehydrated by successive incubations in increasing concentrations of ethanol, and embedded in TAAB 812 resin (Marivac, Halifax, Nova Scotia, Canada). Ultrathin sections were cut using a Ultracut Microtome (Reichert-Jung) and examined in a Phillips 410 electron microscope.

**Morphometric Analysis of Peroxisomes**—Electron micrographs were enlarged to a ×16,000 magnification on photographic paper. Images of cells were cut out and weighed, and the total cell area was calculated using standards (weights of pieces of photographic paper of known area). To quantify peroxisome size, the average peroxisome area was calculated. Essentially, peroxisome profiles were cut out and weighed, and the total peroxisome area was calculated and divided by the total number of peroxisomes counted. To quantify peroxisome number, the numerical density of peroxisomes (number of peroxisomes per μm2 of cell volume) was calculated by the method of Weibel and Bolender (24) for spherical organelles as follows. First, the total number of peroxisome profiles was counted and reported as the number of peroxisomes per cell area (Np). Next, the total volume density (Nv) was calculated for each strain (total peroxisome area/total cell area assayed). Using the values Np and Nv, the numerical density of peroxisomes was determined (24).

**Site-directed Mutagenesis of the MFE2 Gene**—A two-step PCR procedure was used to generate a modified MFE2 gene encoding Myc-MFE2AAB, a Myc-tagged version of MFE2 containing the mutations G16S and G324S. A portion of the MFE2 gene 5′ to the nucleotides encoding Gly-16 was amplified from pG7Myc-MFE2 with oligonucleotides 1015 and 1016 (Table II). The two products were used as templates for a second round of PCR with oligonucleotides 901 and 913. The second PCR product containing the G16S mutation and the total peroxisome area was calculated and divided by the total number of peroxisomes counted. To quantify peroxisome number, the numerical density of peroxisomes (number of peroxisomes per μm2 of cell volume) was calculated by the method of Weibel and Bolender (24) for spherical organelles as follows. First, the total number of peroxisome profiles was counted and reported as the number of peroxisomes per cell area (Np). Next, the total volume density (Nv) was calculated for each strain (total peroxisome area/total cell area assayed). Using the values Np and Nv, the numerical density of peroxisomes was determined (24).

**RESULTS**

**Isolation and Characterization of the Y. lipolytica MFE2 Gene**—Sequencing of a DNA fragment recovered from a Y. lipolytica strain that contained an incorrectly integrated PEX16 disruption construct and that failed to grow on oleic acid-containing medium led to the identification of a 1146-bp piece of the MFE2 gene encoding peroxisomal β-oxidation MFE2 of Y. lipolytica. This DNA sequence was used to generate a probe to isolate the entire Y. lipolytica MFE2 gene by colony hybridization. Three positively hybridizing colonies were identified out of 5000 colonies screened, and the plasmids recovered from each positive colony were shown to be identical by restriction mapping. Sequencing demonstrated that the insert contained the entire MFE2 ORF, as well as 3474 and 426 bp upstream and downstream, respectively, of the ORF.

**Overexpression of the POT1 Gene Encoding Peroxisomal Thiolase**—An overexpression cassette containing the promoter and terminator regions of the ACT1 gene encoding Y. lipolytica act was constructed using a two-step PCR procedure. The 1045 bp upstream of the ACT1 ORF was amplified from Y. lipolytica genomic DNA with oligonucleotides 1009 and 1012 (Table II). 462 bp downstream of the ACT1 ORF was amplified with oligonucleotides 1010 and 1011 (Table II). The two products were used as templates for a second round of PCR with oligonucleotides 1015 and 1016. The PCR product containing G46A was digested with EcoRV and NcoI and ligated into the corresponding sites of pG7Myc-MFE2 to yield the plasmid pG7Myc-MFE2A. Next, the PCR product containing the G1045A and A1047T mutations was digested with EcoRV and AatII and ligated into the corresponding sites of pG7Myc-MFE2A to yield pG7Myc-MFE2ΔΔ. pG7Myc-MFE2AΔΔ was digested with SpI, and the fragment containing MFE2 ORF, as well as 366 and 426 bp upstream and downstream of the MFE2 gene, respectively, was ligated to the SpI site of pNLA445 to generate pMyc-MFE2ΔΔ.

**Overexpression of the POT1 Gene Encoding Peroxisomal Thiolase**—An overexpression cassette containing the promoter and terminator regions of the ACT1 gene encoding Y. lipolytica thiolase was used to overexpress the Y. lipolytica MFE2 gene. The relative difference in the levels of thiolase in the pTHI-OV transformed strain versus the wild-type strain was determined by a comparison of the immunosignals for thiolase from different dilutions of lysate of each strain.

**Antibodies**—Guinea pig antibodies to Y. lipolytica thiolase and rabbit antibodies to Y. lipolytica acyl-CoA oxidase were kindly donated by Jean-Marc Nieud (Laboratoire de Genetique des Microorganismes, Thivar-Margob, France) and were described previously (25). Rabbit antibodies to Y. lipolytica Pex16p were described previously (16). Guinea pig antibodies to Y. lipolytica Pex16p were a kind gift of Gareth Lambkin (Department of Cell Biology, University of Alberta).

Miscellaneous—Oligonucleotides were synthesized on an Oligo 1000M DNA synthesizer (Beckman). Sequencing was performed with an ABI Prism 310 genetic analyzer (Applied Biosystems). DNA was amplified using Ready To Go PCR beads (Amersham Pharmacia Biotech). Protein alignments were done using the Clustal W algorithm of Omiga 1.1.3 (Oxford Molecular, Campbell, CA). Preparation of yeast lysates by disruption with glass beads, growth of E. coli, and manipulation of DNA and RNA were performed as described (28). Protein concentrations were determined using a commercially available kit (Bio-Rad). SDS-PAGE was performed essentially as described (29). Proteins were transferred to nitrocellulose for immunoblotting using a wet transfer system (30), and antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**RESULTS**

**Isolation and Characterization of the Y. lipolytica MFE2 Gene**—Sequencing of a DNA fragment recovered from a Y. lipolytica strain that contained an incorrectly integrated PEX16 disruption construct and that failed to grow on oleic acid-containing medium led to the identification of a 1146-bp piece of the MFE2 gene encoding peroxisomal β-oxidation MFE2 of Y. lipolytica. This DNA sequence was used to generate a probe to isolate the entire Y. lipolytica MFE2 gene by colony hybridization. Three positively hybridizing colonies were identified out of 5000 colonies screened, and the plasmids recovered from each positive colony were shown to be identical by restriction mapping. Sequencing demonstrated that the insert contained the entire MFE2 ORF, as well as 3474 and 426 bp upstream and downstream, respectively, of the ORF.

The Y. lipolytica MFE2 gene and its encoded protein, MFE2, are shown in Fig. 1. A putative TATA box, TATGATAA, is found between nucleotides −124 and −118 (where +1 is the “A” nucleotide of the putative initiation codon of the MFE2 gene). “A” nucleotides are found at positions −1 and −3 upstream of the initiator ATG, a feature common to strongly expressed genes of Y. lipolytica (31). The upstream regulatory region of the MFE2 gene contains the sequence CGGTNATTA. The three nucleotides −495 and −467. This sequence closely resembles the consensus oleic acid-response element (C/T)(G/GTTAG/TTCAG/CAG) of Candida tropicalis (32) and fits the consensus oleic acid-response element CGGNNTTA of S. cerevisiae (33, 34), which are sequences often found upstream of genes encoding peroxisomal matrix proteins. The MFE2 gene contains an intron. Y. lipolytica intron donor and branch sites (31) were identified between nucleotides 172 and 178 and between nucleotides 234 and 245, respectively. The donor site GTGAGTA fits the consensus donor site GTGAGTPu, whereas the sequence AACTAAACCCG resembles very closely the consensus branch site (T/C)/A/G/TAA/C/N,3/CAG. The S. cerevisiae transcription termination consensus sequence tag TAG ... TAT/G ... TTT located upstream of poly(A) addition sites (35) is also a feature of Y. lipolytica genes (31) and is found in the 3′ untranslated region of the MFE2.
of the MFE2 gene. A eukaryotic poly(A) addition signal, AATAAA (36), is found between nucleotides 3113 and 3118. The protein deduced from the MFE2 nucleotide sequence is 901 amino acid residues in length and has a predicted molecular mass of 97,300 Da. A search of protein databases using the BLAST algorithm available through the network service of the National Center for Biotechnology Information showed that the encoded protein has a high degree of homology with peroxisomal MFE2 enzymes of other fungi (Fig. 2). The highest homology is with \textit{Neurospora crassa} MFE2, which shares 62% identical residues and 10% similar residues with \textit{Y. lipolytica} MFE2. The carboxyl-terminal tripeptide Ala-Lys-Leu of \textit{Y. lipolytica} MFE2 fits the PTS1 consensus sequence.

The entire MFE2 ORF was deleted by integration of the \textit{Y. lipolytica} \textit{URA3} gene into the \textit{E122} wild-type strain to create the strain \textit{mfe2-KO}. The \textit{mfe2-KO} strain grew well on media containing acetate (see Fig. 8C) or glucose (data not shown), which do not require peroxisomal \(\beta\)-oxidation for their metabolism, but failed to grow on medium containing oleic acid as the carbon source, which does require peroxisomal \(\beta\)-oxidation for its metabolism (Fig. 3). Transformation of the \textit{mfe2-KO} strain with the MFE2 expression plasmid, pMFE2, led to the restoration of growth on oleic acid medium (Fig. 3).

Synthesis of MFE2 Is Induced by Growth of \textit{Y. lipolytica} in Oleic Acid Medium—Because a putative oleic acid-response element is present upstream of the MFE2 gene, we determined what effects growth of \textit{Y. lipolytica} in oleic acid medium had on the synthesis of MFE2. To monitor the presence of MFE2, the MFE2 gene was modified to encode Myc-MFE2, a version of the protein tagged at its amino terminus with the human c-Myc epitope. Cells of the \textit{mfe2-KO} strain transformed with a plasmid encoding Myc-MFE2 were able to grow on oleic-acid medium (Fig. 3), indicating that Myc-MFE2 functions as does the wild-type MFE2 protein. Anti-c-Myc antibodies detected a protein in extracts of wild-type cells transformed with the plasmid pMyc-MFE2 encoding Myc-MFE2 but not in extracts of wild-type cells transformed with the parental plasmid pINA445 (Fig. 4). Myc-MFE2 was much more abundant in an extract of oleic acid-grown cells than in an extract of glucose-grown cells, whereas the levels of an endoplasmic reticulum protein, Kar2p, were unaffected by the composition of the growth medium (Fig. 4).

Myc-Tagged MFE2 Is Peroxisomal in Wild-type Cells but Is Cytosolic in a \textit{PEX5} Gene Disruption Strain—Double-label, indirect immunofluorescence microscopy was performed on oleic-acid-grown strains transformed with pMyc-MFE2 to de-
termine the subcellular localization of MFE2. Myc-MFE2 had a punctate localization that was identical to that of the peroxisomal matrix enzyme thiolase in both wild-type cells and mfe2-KO cells (Fig. 5A), indicating that Myc-MFE2 is peroxisomal. Because MFE2 shows a typical PTS1 motif, Ala-Lys-Leu, at its carboxyl terminus, we examined the localization of Myc-MFE2 in the strain pex5-KO2, which does not synthesize the PTS1 receptor. Myc-MFE2 gave a diffuse pattern of fluorescence characteristic of a cytosolic localization in the pex5-KO2 strain, whereas thiolase, which is targeted by a PTS2-mediated pathway, still showed a punctate pattern of fluorescence characteristic of peroxisomes (Fig. 5B). Therefore, Myc-MFE2 requires the PTS1 receptor for its targeting to peroxisomes.

The Carboxyl-terminal Tripeptide Ala-Lys-Leu Is Necessary for Targeting Myc-MFE2 to Peroxisomes but Not in the Presence of Full-length MFE2—The data presented above are consistent with the carboxyl-terminal tripeptide Ala-Lys-Leu of MFE2 acting as a PTS1. To test whether this tripeptide is a PTS1, a mutant MFE2 gene encoding a carboxyl-terminally truncated, Myc-tagged version of MFE2, Myc-MFE2ΔALK, was transformed into both the mfe2-KO and wild-type strains. The mutant MFE2 gene was unable to rescue growth of mfe2-KO cells on oleic acid-containing medium (Fig. 3). To determine the localization of Myc-MFE2ΔALK in both strains, double-labeling immunofluorescence microscopy was performed. In mfe2-KO cells, the truncated protein had a cytosolic localization, whereas thiolase had a punctate localization characteristic of peroxisomes (Fig. 5B). These data indicate that the carboxyl-terminal Ala-Lys-Leu of MFE2 is a PTS1, as it is essential for the targeting of Myc-MFE2 to peroxisomes.

Next we determined the localization of MFE2ΔALK in wild-type cells. Interestingly, although Myc-MFE2ΔALK showed a cytosolic localization in mfe2-KO cells, it showed a punctate pattern of distribution in wild-type cells that was identical to the distribution of peroxisomal thiolase (Fig. 5B). These results are consistent with the import of MFE2 as an oligomer into peroxisomes. In a manner similar to what has been shown for the import of the thiolase dimer in S. cerevisiae (7), the carboxyl-terminally truncated form of Myc-MFE2 lacking its PTS1 may heterodimerize with full-length MFE2 encoded in the nucleus and then be targeted to the peroxisome by virtue of the PTS1 of the full-length MFE2. Although it is unknown whether Y. lipolytica MFE2 forms an oligomer, it is important to note that MFE2 of S. cerevisiae has been shown to form homodimers (3).

Peroxisomes of Oleic Acid-induced mfe2-KO Cells Are Larger and More Numerous Than Those of the Wild-Type Strain—Previous evidence has suggested that human MFE2 has a role in controlling peroxisome abundance and size (19). We therefore investigated the effects of disrupting the Y. lipolytica MFE2 gene on peroxisome morphology. Electron microscopy showed that the peroxisomes of mfe2-KO cells appeared more variable in size and more abundant than peroxisomes of wild-type cells (Fig. 6A). Also, the peroxisomes of the mutant strain were often clustered, whereas the peroxisomes of the wild-type strain were usually well separated (Fig. 6A). Morphometric analysis (24) showed that mfe2-KO cells had a numerical den-
FIG. 3. Growth of *Y. lipolytica* strains on oleic acid-containing medium. The wild-type strain *E122* and the deletion strain *mfe2-KO*, transformed with the plasmids indicated, were grown in YND medium overnight. Cells were harvested by centrifugation, washed, resuspended in water, spotted onto YNO agar plates and grown for 2 days.

FIG. 4. Synthesis of Myc-MFE2 in *Y. lipolytica* in oleic acid-containing medium. Cells of the wild-type strain *E122* transformed with either pMycMFE2 encoding Myc-MFE2 or the parental plasmid pINA445 were grown overnight in YND medium, harvested by centrifugation, transferred to fresh YND or YNO medium, and grown for an additional 9 h. Cells were harvested, and lysates were prepared by disruption with glass beads. Equal amounts of protein from each strain under each growth condition were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-c-Myc antibodies (top panel) or anti-Kar2p antibodies (bottom panel). The numbers at the left indicate the migrations of molecular mass standards (in kDa).

sity of peroxisomes that was approximately 2.5 times that of wild-type cells (Table III, rows 1 and 2). Peroxisomes of the *mfe2-KO* strain were also much more variable in size than those of wild-type cells, with mutant cells having many peroxisomes greater than 0.4 µm² in area and wild-type cells having essentially no peroxisomes greater than 0.4 µm² in area (Fig. 6B). In addition, the average area of *mfe2-KO* peroxisomes was significantly larger than the average area of *E122* peroxisomes (Table III, rows 1 and 2). Using the Student two-sample *t* test for unequal variance, the average peroxisome area of *mfe2-KO* cells was found to be 1.26 ± 0.07 times larger than that of wild-type cells, with a 95% confidence interval.

The fact that peroxisomes are more abundant in the *mfe2-KO* strain than in the wild-type strain was not obvious from immunofluorescence microscopy (Fig. 5B). This apparent difference between electron microscopic and immunofluorescence microscopic analyses can be explained by the fact that peroxisomes of the deletion strain are often clustered (Fig. 6A), and a cluster of peroxisomes can appear as one large peroxisome in immunofluorescence microscopy (37).

*mfe2-KO* Cells Have Enlarged Peroxisomes When Grown on a Carbon Source Not Metabolized by Peroxisomal β-Oxidation—Our morphological analysis of oleic acid-induced *mfe2-KO* cells suggested a dysregulation of peroxisome size and number in the absence of MFE2. To better understand the mechanism responsible for this observation, we investigated the morphology of *mfe2-KO* cells grown in medium containing acetate, a carbon source not requiring β-oxidation for its metabolism. Peroxisomes of *mfe2-KO* cells grown in acetate were considerably larger than those of wild-type cells in electron micrographs (Fig. 7). Morphometric analysis of acetate-grown strains showed that the average area of peroxisomes of *mfe2-KO* cells was 3.2 times that of wild-type cells, whereas the number of peroxisomes counted per µm² of cell area was similar for both strains (Table III, rows 3 and 4). However, a similar peroxisome count does not necessarily mean that the average number of peroxisomes per cell is the same for both strains, because the larger a peroxisome is, the greater the probability of observing it in any given micrograph (24). To estimate the peroxisome number for cells of each strain, we calculated the peroxisome numerical density, a value that takes into account peroxisome size (24). This calculation showed that in contrast to what was observed for cells grown in...
Control of Peroxisome Size and Number in Yeast

FIG. 6. Peroxisomes are larger, more abundant and more variable in size in mfe2-KO cells than in wild-type cells. A, ultrastructure of mutant mfe2-KO and wild-type E122 cells. Cells were grown in YEPD medium overnight, transferred to YPBO medium, and grown in YPBO medium for 14 h. Cells were fixed and processed for electron microscopy. P, peroxisome; M, mitochondrion; N, nucleus; V, vacuole. Bar, 1 μm. B, morphometric analysis of oleic acid-grown E122 (left panel) and mfe2-KO (right panel) cells. Electron micrographs encompassing 825 μm² of wild-type cell area and 553 μm² of mfe2-KO cell area were enlarged to a magnification of ×16,620 on photographic paper. Peroxisome profiles were cut out and weighed, and the total peroxisome area for each strain was calculated. Next, the areas of individual peroxisomes were calculated, and peroxisomes were separated into size categories. A histogram was generated for each strain depicting the percentage of total peroxisome area occupied by the peroxisomes of each category. The numbers along the x axis are the maximum sizes of peroxisomes in each category (in μm²).

oleic acid, mfe2-KO cells contained approximately half as many peroxisomes as did wild-type cells following growth in acetate (Table III, rows 3 and 4).

Enlarged Peroxisomes Are Not Caused by the Absence of MFE2 Protein—To determine whether the enlarged peroxisomes present in mfe2-KO cells were caused by the absence of MFE2 protein or the lack of a functional β-oxidation pathway, we determined whether a nonfunctional version of Myc-MFE2 could reverse the morphological defects of the mfe2-KO strain. S. cerevisiae MFE2 has two (3R)-hydroxyacyl-CoA dehydrogenase domains (DA and DB in Fig. 8A) that are required for the β-oxidation of fatty acids (4). A form of MFE2 containing the mutations G16S and G324S in the nucleotide-binding sites of the two dehydrogenase domains has been shown to be nonfunctional in peroxisomal β-oxidation (4). We identified two consensus nucleotide-binding sites in Y. lipolytica MFE2, and alignment of the S. cerevisiae and Y. lipolytica nucleotide-binding sites revealed that Gly-16 and Gly-324 of Y. lipolytica MFE2 correspond to Gly-16 and Gly-329 of S. cerevisiae MFE2, respectively (Fig. 8B). The plasmid pMyc-MFE2ΔΔAB expressing Myc-MFE2ΔΔAB, which contains the mutations G16S and G324S, was constructed and transformed into mfe2-KO cells. The mfe2-KO strain transformed with pMyc-MFE2ΔΔAB, like the mfe2-KO strain transformed with the empty plasmid pINA445, was unable to grow on agar medium containing oleic acid as the carbon source, whereas the mfe2-KO strain transformed with a plasmid expressing Myc-MFE2 grew well on this medium (Fig. 8C, second panel). All three strains grew well on agar medium containing acetate as the carbon source (Fig. 8C, top panel). Myc-MFE2ΔΔAB and Myc-MFE were detected by immunoblot analysis of yeast lysates with anti-c-Myc antibodies. The levels of Myc-MFE2ΔΔAB and Myc-MFE2 were similar in the yeast lysates in which they were expressed (Fig. 8C, third panel). The levels of the endoplasmic reticulum protein Kar2p were similar in the lysates of all three strains, confirming that equal amounts of total protein were analyzed (Fig. 8C, bottom panel). Thus, Myc-MFE2ΔΔAB is unable to restore fatty acid β-oxidation activity to the mfe2-KO strain. When grown on acetate-containing medium, the mfe2-KO strain transformed with pMyc-MFE2ΔΔAB, like the mfe2-KO strain transformed with the empty plasmid pINA445, had peroxisomes larger than those of the mfe2-KO strain transformed with pMyc-MFE2, which encodes a functional version of MFE2 (Fig. 9). The average area of peroxisomes was determined for each strain (Table III, rows 5–7). Peroxisomes of the mfe2-KO strain synthesizing Myc-MFE2ΔΔAB were larger than peroxisomes of the wild-type strain, but not as large as peroxisomes of the disruption strain. Because the mfe2-KO strain expressing normal levels of a nonfunctional form of Myc-MFE2 still contained enlarged peroxisomes, the presence of enlarged peroxisomes in the mfe2-KO strain was not the result of the absence of MFE2 protein.

β-Oxidation Enzyme Levels Are Increased in the mfe2-KO Strain—An analysis of the levels of several peroxisomal proteins in total cell lysates of the mutant and wild-type strains grown in glucose-, oleic acid-, or acetate-containing medium showed that two peroxisomal β-oxidation enzymes, thiolase and acyl-CoA oxidase 5 (one of five Y. lipolytica acyl-CoA oxidase isoenzymes (27)), were noticeably increased in mfe2-KO cells as compared with wild-type cells by growth in each carbon source (Fig. 10). The levels of two peroxisomal enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, appeared unchanged from the levels in wild-type cells or were marginally increased in mfe2-KO cells. The levels of two peroxins that associate with the peroxisome membrane, Pex16p and Pex19p, were not increased in mfe2-KO cells compared with wild-type cells, and in fact, both peroxins appeared less abundant in the deletion strain than in the wild-type strain following growth in oleic acid. The levels of the endoplasmic reticulum protein POT1, the gene encoding peroxisomal thiolase, in wild-type cells and analyzed the effect of this overexpression on peroxisome size. We looked at peroxisome size after growth of cells in glucose-containing medium, because the level of...
Control of Peroxisome Size and Number in Yeast

endogenous thiolase is very low under this condition (Fig. 10). Therefore, a high relative increase in the abundance of thiolase can be readily achieved by overexpression of its gene in wild-type cells grown in glucose. Also, because peroxisomes are normally very small in cells grown in glucose (Fig. 11A), the relative increase in the abundance of thiolase led to peroxisomes in the pTHI-OV transformed strain (Fig. 11B, top panel) that were noticeably larger than those of the pINA445 transformed strain (Fig. 11B, bottom panel). The average area and the numerical density of peroxisomes was determined for each strain (Table III, rows 8 and 9). Peroxisomes of the strain overexpressing POT1 were almost twice as large as peroxisomes of the wild-type strain, whereas the numerical density of peroxisomes was similar for both strains. These results suggest that an increase in abun-

### Table III

| Row number | Strain          | Growth medium | Cell area assayed | Peroxisome count | Numerical density of peroxisomes | Average area of peroxisomes |
|------------|-----------------|---------------|-------------------|------------------|----------------------------------|----------------------------|
| 1          | E122            | YPBO          | 825               | 0.26             | 1.48                             | 0.145 ± 0.057              |
| 2          | mfe2-KO         | YPBO          | 550               | 0.74             | 1.20                             | 0.150 ± 0.109              |
| 3          | E122            | YEPA          | 725               | 0.11             | 0.38                             | 0.075          |
| 4          | mfe2-KO         | YEPA          | 880               | 0.09             | 0.19                             | 0.243          |
| 5          | mfe2-KO + pMyc-MFE2 | YNA         | 447               | 0.14             | 0.32                             | 0.103          |
| 6          | mfe2-KO + pINA445 | YNA         | 264               | 0.13             | 0.18                             | 0.267          |
| 7          | mfe2-KO + pMyc-MFE2ΔΔB | YNA       | 634               | 0.15             | 0.26                             | 0.171          |
| 8          | E122 + pINA445  | YND           | 629               | 0.14             | 0.49                             | 0.045           |
| 9          | E122 + pTHI-OV  | YND           | 475               | 0.21             | 0.51                             | 0.087           |

a Number of peroxisomes counted per μm² of cell area on micrographs.

b Number of peroxisomes per μm² of cell volume (24).

c Average area on micrographs.

d Standard error was not calculated as peroxisomes were not measured individually.

**Fig. 7.** Peroxisomes of mfe2-KO cells are larger than those of wild-type cells when grown in acetate-containing medium. Ultrastructure of mfe2-KO and E122 cells grown in acetate-containing YEPA medium for 24 h. Cells were fixed and processed for electron microscopy. P, peroxisome; M, mitochondrion; N, nucleus; V, vacuole. Bar, 1 μm.

**Fig. 8.** Expression of Myc-MFE2ΔΔB does not restore growth on oleic acid medium to the mfe2-KO strain. A, domains of yeast MFE2. The two (3R)-hydroxyacyl-CoA dehydrogenase domains (DA and DB) are represented by solid lines. The 2-enoyl-CoA hydratase 2 domain (D) is represented by an open box. Nucleotide-binding sites in the dehydrogenase domains are represented by closed boxes. B, sequence alignment of the nucleotide-binding sites of *S. cerevisiae* and *Y. lipolytica* MFE2 proteins. Residues identical or similar in three or more sequences are indicated in boldface. Similarity rules: A = S = T; D = E; N = Q; R = K; L = M = V; F = Y = W. Conserved glycine residues mutated in Mfe-MFE2ΔΔB are boxed. Residues are numbered at the right. C, growth characteristics and protein profiles of the mfe2-KO strain transformed with pMyc-MFE2ΔΔB, pMyc-MFE2, or pINA445. Strains were grown as described in the legend to Fig. 3 and spotted onto YNA agar (top panel) and YNO agar (second panel from top). Strains were also grown overnight in YND medium, transferred to YNO medium, and grown for an additional 10 h in YNO medium. Cells were harvested, and lysates were prepared by disruption with glass beads. Equal amounts of protein from each strain were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-c-Myc antibodies (third panel from top) or anti-Kar2p antibodies (bottom panel).
dance of peroxisomal \( \beta \)-oxidation enzymes in the mfe2-KO strain contributes to the enlarged peroxisomes seen in this strain.

DISCUSSION

The MFE2 gene of \( Y. \) lipolytica encodes peroxisomal MFE2, which shares a high degree of sequence homology with MFE2 proteins from other fungi. \( Y. \) lipolytica cells devoid of MFE2 are unable to utilize oleic acid as a carbon source, as has been observed for \( S. \) cerevisiae (4). The \( Y. \) lipolytica MFE2 gene contains a putative upstream oleic acid-response element, as do the genes of \( S. \) cerevisiae (33, 34) and \( C. \) tropicalis (32), and consistent with this, the synthesis of Myc-MFE2, a c-Myc epitope-tagged version of the protein, was induced by growth of \( Y. \) lipolytica on oleic acid-containing medium. \( Y. \) lipolytica MFE2 contains a PTS1, as do the MFE2 proteins of \( C. \) tropicalis and \( S. \) cerevisiae, and it is essential for targeting to peroxisomes. Similar to \( S. \) cerevisiae MFE2, which has been shown to form homodimers (3), our evidence suggests that \( Y. \) lipolytica MFE2 is targeted to peroxisomes as an oligomer.

\( Y. \) lipolytica MFE2 appears to have a role in peroxisome biogenesis. Cells of the deletion strain mfe2-KO grown in oleic acid contain more and larger peroxisomes than do wild-type cells grown under the same conditions. Also, peroxisomes of the mfe2-KO strain are more heterogeneous in size than those of wild-type cells and are often found in close proximity to one other. mfe2-KO cells grown in acetate, a carbon source not requiring peroxisomes for its metabolism, have larger peroxisomes than do identically grown wild-type cells, but only half as many. These data suggest that MFE2 contributes to the regulation of peroxisome size and number.

mfe2-KO cells contain enlarged peroxisomes in the presence of both oleic acid and acetate carbon sources. Consistent with our observations in yeast, peroxisomes are enlarged in a mammalian cell line lacking MFE2 (19). The larger size of mfe2-KO peroxisomes may be the result of increased amounts of other peroxisomal \( \beta \)-oxidation enzymes in this strain. It has been shown previously that high expression of genes encoding matrix enzymes in cells of \( H. \) polymorpha and \( S. \) cerevisiae leads to enlarged peroxisomes (38–40). Here, we show that increasing the amount of thiolase in \( Y. \) lipolytica cells leads to enlarged peroxisomes, indicating that increased levels of \( \beta \)-oxidation proteins in mfe2-KO cells may contribute to the enlarged peroxisomes seen in this strain, although, interestingly, similar levels of Myc-MFE2ΔΔB and Myc-MFE2 (Fig. 8C) resulted in smaller peroxisomes being seen in the mfe2-KO strain expressing pMyc-MFE2ΔΔB relative to mfe2-KO strain transformed with the parental plasmid pINA445 alone (see Table III, rows 7 and 6 respectively). Additionally, we have shown that the increase in peroxisome size seen for mfe2-KO cells is not due to the absence of MFE2 protein, as a nonfunctional form of MFE2 expressed at normal MFE2 levels is unable to complement this
following growth in oleic acid, mfe2-KO somes than do wild-type cells. In contrast, contrast to wild-type cells, cells of the oxisomes within a cell may be affected by the rate of cell possible reason for this dichotomy is that the number of peroxisomes per cell of acetate have fewer peroxisomes than do wild-type cells. A morphological defect. Therefore, it appears that the inability of MFE2 to function in fatty acid -oxidation leads to enlarged peroxisomes. Deletion of the MFE2 gene affects peroxisome number. Following growth in oleic acid, mfe2-KO cells have more peroxisomes than do wild-type cells. In contrast, mfe2-KO cells grown in acetate have fewer peroxisomes than do wild-type cells. A possible reason for this dichotomy is that the number of peroxisomes within a cell may be affected by the rate of cell division. It has been proposed that the number of peroxisomes in a cell is the result of both peroxisome accumulation due to proliferation and peroxisome loss due to cell division (41). In contrast to wild-type cells, cells of the mfe2-KO deletion strain cannot readily divide in oleic acid-containing medium, so few of their peroxisomes segregate to budding daughter cells. If peroxisomes continue to proliferate in mfe2-KO cells in the absence of cell division, possibly because of the inability of the strain to metabolize oleic acid, peroxisomes will accumulate in mother cells of the mfe2-KO strain, thereby effectively increasing the number of peroxisomes per cell. Consistent with this scenario is the fact that in acetate, mfe2-KO cells divide at the rate of wild-type cells and do not have more peroxisomes than do wild-type cells. An alternative possibility for the increased number of peroxisomes in the oleic acid-grown mfe2-KO cells is that disruption of the MFE2 gene causes an increased rate of peroxisome proliferation under these conditions. However, how this would occur is not readily apparent. Nevertheless, deletion of the MFE2 gene does affect the number of peroxisomes in Y. lipolytica, whether due to a secondary effect of cell division or a primary effect of peroxisome proliferation.

It should be noted that although peroxisomes in the mfe2-KO strain are 2.5 times more abundant than, or half as abundant as, peroxisomes of the wild-type strain (depending on the growth condition), Chang et al. (19) observed that peroxisomes are 5-fold less abundant in human cells lacking MFE2 than in normal cells. One reason for the difference between yeast and human cells may be the method of analysis in that the human peroxisomes were observed using only immunofluorescence microscopy. If peroxisomes in the mutant human cells are clustered as they are in oleic acid-induced mfe2-KO cells, the number of peroxisomes may have been underestimated by using immunofluorescence microscopy. Alternatively, this difference in peroxisome number between yeast and human cells may indicate a difference in the mechanisms regulating peroxisome abundance in these two systems. Future studies will be aimed at further determining the molecular players and events involved in these mechanisms.

Acknowledgment—We thank Honey Chan for technical assistance with electron microscopy.

REFERENCES

1. Hashimoto, T. (1999) Neurochem. Res. 24, 551–563
2. Endrizzi, A., Pagot, Y., Le Clainche, A., Nicaud, J.-M., and Belin, J.-M. (1996) Crit. Rev. Biotechnol. 16, 301–329
3. Hiltunen, J. K., Wenzel, B., Beyer, A., Erdmann, R., Fosså, A., and Kunau, W. H. (1992) J. Biol. Chem. 267, 6646–6653
4. Qin, Y.-M., Marttila, M. S., Haapalainen, A. M., Sivari, K. M., Glumoff, T., and Hiltunen, J. K. (1999) J. Biol. Chem. 274, 29619–29625
5. Subramani, S. (1998) Physiol. Rev. 78, 171–188
6. Hettema, E. H., Distel, B., and Tabak, H. F. (1999) Biochim. Biophys. Acta 1451, 17–34
7. Glover, J. R., Andrews, D. W., and Rachubinski, R. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10541–10545
8. McNew, J. A., and Goodman, J. M. (1994) J. Cell Biol. 127, 1245–1257
9. Tietz, V. I., Smith, J. J., Szilard, R. K., and Rachubinski, R. A. (1998) J. Cell Biol. 142, 403–420
10. van den Bosch, H., Schutgens, R. B. H., Wanders, R. J. A., and Tager, J. M. (1999) Annu. Rev. Biochem. 61, 157–197
11. Tan, X., Waterham, H. R., Veenhuis, M., Cregg, J. M. (1995) J. Cell Biol. 128, 307–319
12. Erdmann, R., and Blebel, G. (1995) J. Cell Biol. 129, 509–523
13. Marshall, P. A., Krinkevich, V. I., Lark, R. J., Dyer, J. M., Veenhuis, M., and Goodman, J. M. (1995) J. Cell Biol. 129, 345–355
14. Sakai, Y., Marshall, P. A., Saigani, A., Takabe, K., Saki, H., Kato, N., and Goodman, J. M. (1995) J. Bacteriol. 177, 6773–6781
15. Marshall, P. A., Dyer, J. M., Quick, M. E., and Goodman, J. M. (1996) J. Cell Biol. 135, 123–136
16. Eltzen, G. A., Szilard, R. K., and Rachubinski, R. A. (1997) J. Cell Biol. 137, 1265–1278
17. Schrader, M., Reuber, B. E., Morrell, J. C., Jimenez-Sanchez, G., Obie, C., Stroh, T. A., Valle, D., Schroer, T. A., and Gould, S. J. (1998) J. Biol. Chem. 273, 29607–29614
18. Poll-The, B. T., Roels, P., Ogier, H., Scott, J., Vamecq, J., Schutgens, R. B. H., Wanders, R. J. A., van der Velde, C. W. T., van Wijland, M. J. A., Schram, A. W., Rutgers, J. M., and Saoudhary, J.-M. (1988) Am. J. Hum. Genet. 42, 422–434
19. Chang, C.-C., South, S., Warren, D., Jones, J., Moser, A. B., Moser, H. W., and Gould, S. J. (1999) J. Cell Biol. 145, 1579–1590
20. Nuttley, W. M., Brade, A. M., Gassmann, C., Eltzen, G. A., Glover, J. R., Aitchison, J. D., and Rachubinski, R. A. (1993) Yeast 9, 507–517
21. Szilard, R., Tietz, V. I., Veenhuis, M., and Rachubinski, R. A. (1995) J. Bacteriol. 173, 1453–1460
22. Kolodziej, P. A., and Young, R. A. (1991) Methods Enzymol. 194, 508–519
23. Pringle, J. R., Adams, A. E. M., Drubin, D. G., and Haarer, B. K. (1991) Methods Enzymol. 194, 565–592
24. Weibel, E. R., and Bolender, P. (1973) in Principles and Techniques of Electron Microscopy (Hayat, M. A. ed). Van Nostrand Reinhold, New York
25. Eltzen, G. A., Tietz, V. I., Smith, J. J., Veenhuis, M., Szilard, R. K., and Rachubinski, R. A. (1996) J. Biol. Chem. 271, 20300–20306
26. Tietz, V. I., Ogrydziak, D. M., and Rachubinski, R. A. (1997) Mol. Cell. Biol. 17, 5210–5226

A

![Diagram of peroxisomes](https://example.com/diagram.png)

**FIG. 11.** Increased levels of the oxidation enzyme thiolase lead to enlarged peroxisomes. Wild-type E122 cells transformed with either the POT1 overexpression plasmid pTHI-OV or the parental plasmid pNA445 were grown in glucose-containing YND medium overnight. Analysis of the cells was performed by electron microscopy. A, peroxisome; M, mitochondrion; N, nucleus. Bar, 1 μm.

B

![Diagram of peroxisomes](https://example.com/diagram.png)
27. Wang, H. J., Le Dall, M.-T., Waché, Y., Laroche, C., Belin, J.-M., Gaillardin, C., and Nicaud, J.-M. (1999) *J. Bacteriol.* **181**, 5140–5148
28. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989) *Current Protocols in Molecular Biology*, Greene Publishing Associates, New York
29. Laemmli, U. K. (1970) *Nature* **227**, 680–685
30. Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
31. Barth, G., and Gaillardin, C. (1997) *FEMS Microbiol. Rev.* **19**, 219–237
32. Sloots, J. A., Aitchison, J. D., and Rachubinski, R. A. (1991) *Gene* **105**, 129–134
33. Filipits, M., Simon, M. M., Rapatz, W., Hamilton, B., and Häuß, H. (1993) *Gene* **132**, 49–55
34. Karpichev, I. V., and Small, G. M. (1998) *Mol. Cell. Biol.* **18**, 6560–6570
35. Zaret, K. S., and Sherman, F. (1982) *Cell* **28**, 563–573
36. Proudfoot, N. J., and Brownlee, G. G. (1976) *Nature* **263**, 211–214
37. Zhang, J. W., Han, Y., and Lazarew, P. B. (1993) *J. Cell Biol.* **123**, 1133–1147
38. Distel, B., van der Leij, I., Veenhuis, M., and Tabak, H. F. (1988) *J. Cell Biol.* **107**, 1669–1675
39. Gödecke, A., Veenhuis, M., Roggenkamp, R., Janowicz, Z. A., and Hallenberg, C. P. (1989) *Curr. Genet.* **16**, 13–20
40. Roggenkamp, R., Dition, T., and Kowallik, K. V. (1989) *Mol. Cell. Biol.* **9**, 988–994
41. Veenhuis, M., and Goodman, J. M. (1990) *J. Cell Sci.* **96**, 583–590
