Enlarged Peroxisomes Are Present in Oleic Acid–grown Yarrowia lipolytica Overexpressing the PEX16 Gene Encoding an Intraperoxisomal Peripheral Membrane Peroxin

Gary A. Eitzen, Rachel K. Szilard, and Richard A. Rachubinski
Department of Cell Biology and Anatomy, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Abstract. Pex mutants of the yeast Yarrowia lipolytica are defective in peroxisome assembly. The mutant strain pex16-1 lacks morphologically recognizable peroxisomes. Most peroxisomal proteins are mislocalized to a subcellular fraction enriched for cytosol in pex16 strains, but a subset of peroxisomal proteins is localized at, or near, wild-type levels to a fraction typically enriched for peroxisomes. The PEX16 gene was isolated by functional complementation of the pex16-1 strain and encodes a protein, Pex16p, of 391 amino acids (44,479 D). Pex16p has no known homologues. Pex16p is a peripheral protein located at the matrix face of the peroxisomal membrane. Substitution of the carboxyl-terminal tripeptide Ser-Thr-Leu, which is similar to the consensus sequence of peroxisomal targeting signal 1, does not affect targeting of Pex16p to peroxisomes. Pex16p is synthesized in wild-type cells grown in glucose-containing media, and its levels are modestly increased by growth of cells in oleic acid–containing medium. Overexpression of the PEX16 gene in oleic acid–grown Y. lipolytica leads to the appearance of a small number of enlarged peroxisomes, which contain the normal complement of peroxisomal proteins at levels approaching those of wild-type peroxisomes.

Peroxisomes, along with the glyoxysomes of plants and the glycosomes of trypanosomes, comprise the microbody family of organelles. Peroxisomes compartmentalize a number of essential biochemical functions, including the β-oxidation of fatty acids (Lazarow and de Duve, 1976) and the decomposition of H₂O₂ by catalase (de Duve and Baudhuin, 1966). Peroxisomes are indispensable for normal human development and physiology, as shown by the lethality of genetic disorders such as Zellweger syndrome in which peroxisomes fail to assemble normally (Lazarow and Moser, 1994).

The question of how peroxisomes assemble has received a great deal of attention in recent years. Peroxosomal proteins are synthesized on free polysomes in the cytosol. Most soluble proteins of the matrix are targeted by one of two types of peroxisomal targeting signals (PTS)¹. PTS1 is a carboxyl-terminal tripeptide with the consensus structure (Ser/Ala/Cys)(Lys/Arg/His)(Leu/Met). PTS2 is a sometimes cleaved amino-terminal signal with the consensus structure (Arg/Lys)(Leu/Val/Ile)(X)₅(His/Gln)(Leu/Ala) (for reviews see Subramani, 1993, 1996; Rachubinski and Subramani, 1995). The PTSs of peroxisomal membrane proteins remain less well defined, although Dyer et al. (1996) have recently shown that the membrane protein PMP47 of the yeast Candida boidinii is targeted to peroxisomes by a hydrophilic loop of 20 amino acids located between two membrane-spanning domains. Peroxisomes, and the related glycosomes, are different from mitochondria and chloroplasts in that they can translocate folded and oligomeric proteins (Glover et al., 1994; McNew and Goodman, 1994, 1996; Walton et al., 1995; Häusler et al., 1996).

Complementation of peroxisome assembly mutants, collectively known as pex mutants (Distel et al., 1996), in both yeast and mammalian species has proven invaluable in identifying proteins called peroxins required for peroxisome assembly. Peroxins have been identified that act as receptors for PTS1 or PTS2 motifs (for review see McNew and Goodman, 1996). However, while the analysis of pex mutants has identified an ever-increasing number of peroxins required for peroxisomal protein targeting and import, much less is known about the mechanisms controlling peroxisome size and number. New peroxisomes are thought to arise by fission of preexisting peroxisomes (Lazarow and Fujiki, 1985), and, therefore, peroxisomal templates must be present under all conditions. A peroxisome proliferative response can be elicited in various yeast species by switching from a fermentable carbon source such as glucose to a carbon source that can be metabolized...
solely by peroxisomes, most often oleic acid or methanol. Two models for the temporal order of events of peroxi-
some proliferation have been proposed. In *Hansenula polymorpha*, peroxisomes first grow, and then smaller per-
oxisomes bud off mature peroxisomes (Veenhuis et al.,
1979). In contrast, peroxisome proliferation and fission
precede protein import in *C. boidinii* (Veenhuis and
Goodman, 1990) and most other yeast species (for review
see Subramani, 1993).

A few proteins have been implicated in directly regulat-
ing peroxisome number and size rather than having indi-
crect effects on these processes through general defects in
peroxisomal protein import. Oversynthesis of the peroxi-
somal integral membrane proteins Pex11p of *Saccharomy-
ces cerevisiae* (Marshall et al., 1995) and *C. boidinii* (Sakai
et al., 1995) and Pex3p and Pex10p of *H. polymorpha*
(Baerends et al., 1996; Tan et al., 1995) leads to prolifera-
tion of normal peroxisomes, while cells lacking Pex11p of
*S. cerevisiae* (Erdmann and Blobel, 1995; Marshall et al.,
1995) and *C. boidinii* (Sakai et al., 1995) have fewer but
larger peroxisomes than usual. Here we report the isola-
tion and characterization of the gene *PEX16* of the yeast
*Yarrowia lipolytica* encoding the peroxin Pex16p. Pex16p
is peripherally associated with the matrix face of the
peroxisomal membrane. Interestingly, overexpression of
the *PEX16* gene in oleic acid–grown *Y. lipolytica* results in
the appearance of enlarged peroxisomes that contain the
normal complement of proteins at levels approaching those of
wild-type peroxisomes.

**Materials and Methods**

**Strains, Culture Conditions, and Microbial Techniques**

The *Y. lipolytica* strains used in this study are listed in Table I. Growth was at 30°C. Strains containing plasmids were initially grown in YND (0.67% yeast nitrogen base without amino acids, 2% glucose) medium to an OD
<sub>600</sub> of ~1.5 and then shifted to YNO (0.67% yeast nitrogen base without amino acids, 0.05% [wt/vol] Tween 40, 0.1% [wt/vol] oleic acid) medium to proliferate peroxisomes. Media were supplemented with uracil, leucine, lysine, and histidine each at 50 µg/ml, as required. Strains not containing plasmids were grown as above in YPD (1% yeast extract, 2% peptone, 2% glucose) medium and then shifted to YBPO (0.3% yeast extract, 0.5% peptone, 0.5% K<sub>H</sub>PO<sub>4</sub>, 0.5% KH<sub>2</sub>PO<sub>4</sub>, 1% Brij-35, 1% [wt/
vol] oleic acid) medium to proliferate peroxisomes. DNA manipulation and growth of *Escherichia coli* were performed as previously described (Ausubel et al., 1989).

**Cloning, Sequencing, and Integrative Disruption of the *PEX16* Gene**

The *pex16-1* mutant strain was isolated from randomly mutated *Y. li-
polytica* strain E122*, as previously described (Nuttley et al., 1993). The
*PEX16* gene was isolated by functional complementation of the *pex16-1*
strain using a *Y. lipolytica* genomic DNA library (Nuttley et al., 1993). Leu<sup>+</sup> transformants were replica plated onto selective YNO agar to screen for the ole-
phenotype. Correct integration was confirmed by Southern blot analysis. Disruption strains were crossed with wild-type and *pex16-1* mutant strains, and the resultant diploids were checked for growth on YNO-agar plates.

**Cell Fractionation, Protein Isolation, and Protease Protection**

Total cellular protein was isolated by glass bead disruption of cells in 25
mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.1 mM DTT plus protease inhibitors (0.5 mM PMSF, and pepstatin, chymostatin, antipain, and leupeptin each at 1 µg/ml). Cells were sub-
jected to subcellular fractionation to yield a postnuclear supernatant
(PNS). The PNS was subjected to centrifugation at 20,000 *g<sub>max</sub>* to yield a pellet (20KgP) enriched for peroxisomes and mitochondria and a superna-
tant (20KgS), as previously described (Aitchison et al., 1991). Peroxi-
somes were purified from the 20KgP by isopycnic centrifugation on a dis-
contiguous sucrose gradient (Titorenko et al., 1996). Peroxinsubfractions were prepared by extraction at 4°C for 30 min with one of T18
buffer (10 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.5 mM PMSF), T8 buffer (T8 buffer containing 0.5 M KCl; Erdmann and Blobel, 1995), or carbon-
ate buffer (0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 11.5, 0.5 mM PMSF), followed by centrifugation at 200,000 *g<sub>max</sub>* at 4°C for 30 min in a rotor (TLA 120.2; Beckman Instruments, Fullerton, CA).

Protease protection experiments were performed on 20KgP fractions
isolated in the absence of protease inhibitors. 0, 2, 5, 10, or 25 µg of trypsin
was combined with 200 µg of protein, with or without 0.5% (vol/vol) Trit-
on X-100, in a final volume of 100 µl. Reactions were incubated at 4°C for
30 min and terminated by addition of hot SDS-PAGE sample buffer
(Laemmli, 1970) and immediate boiling. Samples were subjected to SDS-
PAGE, followed by immunoblotting.

**Preparation of Antibodies**

Antibodies to Pex16p were raised in guinea pig and rabbit against a mal-
tose-binding protein–Pex16p fusion. The open reading frame (ORF) of
the *PEX16* gene was amplified from the plasmid p16N1 by PCR, using
primers 249 (5′) and 224 (3′) (Table II). The product was digested with
EcORI and HindIII and ligated into the vector pMAL-c2 (New England Biolabs, Beverly, MA) in-frame and downstream of the gene encoding maltose-binding protein.

Antibodies to acyl-CoA oxidase, isocitrate lyase, thiolase, malate syn-
thase, and Pex2p (previously Pay3p [Eitzen et al., 1996]) were prepared as
previously described (Eitzen et al., 1996). Antibodies to Pex5p (formerly Pay3p [Szilard et al., 1995]) and the carbonyl-terminal SKL-tripeptide
motif were prepared as described (Szilard et al., 1995). 12CA5 mono-

### Table I. *Yarrowia lipolytica* Strains Used in This Study

| Strain | Genotype |
|--------|----------|
| E122*  | Mat<sup>A</sup>, ura-3-302, leu2-270, lys8-11 |
| 22301-3* | Mat<sup>A</sup>, ura-3-302, leu2-270, his1 |
| pex16-1<sup>−</sup> | Mat<sup>A</sup>, ura-302, leu2-270, lys8-11, pex16-1 |
| P16TR<sup>−</sup> | Mat<sup>A</sup>, ura-302, leu2-270, lys8-11, p16HC2(LEU12) |
| P16KO-8<sup>A</sup> | Mat<sup>A</sup>, ura-302, leu2-270, lys8-11, pex16-1 |
| P16KO-8<sup>B</sup> | Mat<sup>A</sup>, ura-302, leu2-270, his1, pex16-1 |
| D16-E1x8B<sup>−</sup> | Mat<sup>A</sup>Amb, ura-302-ura3-302, leu2-270, lys8-11+, +his1, +ipeX16-1: LEU2 |
| D16-8A<sup>−</sup> | Mat<sup>A</sup>Amb, ura-302-ura3-302, leu2-270, lys8-11+, +his1, pex16-1: LEU2+ |
| D16-P16x8B<sup>−</sup> | Mat<sup>A</sup>Amb, ura-302-ura3-302, leu2-270, lys8-11+, +his1, pex16-1: pex16-16: LEU2 |
| D16-P16x22<sup>−</sup> | Mat<sup>A</sup>Amb, ura-302-ura3-302, leu2-270, lys8-11+, +his1, pex16-11+ |
| pex16-HA<sup>−</sup> | Mat<sup>A</sup>, ura-302, leu2-270, lys8-11, pex16-1:: PEX16HA-URA3 |
| pex16-TH<sup>−</sup> | Mat<sup>A</sup>, ura-302::PEX16TH-URA3, leu2-270, lys8-11, pex16-1 |

*G. Gaillardin (Institut National de la Recherche Agronomique-CNRS) Thiverval-
Grignon.

†This study.
clonal antibodies against the influenza virus hemagglutinin (HA) antigen were purchased from the Berkeley Antibody Company (Berkeley, CA).

Immunoblotting and detection of antigen-antibody complexes by enhanced chemiluminescence (Amersham Life Sciences, Mississauga, Ontario, Canada) were performed as described (Szilard et al., 1995).

**Microscopical Analysis**

*Y. lipolytica* cells were harvested 8 h after shifting from glucose-containing medium to oleic acid–containing medium. Whole cells and subcellular fractions were prepared for electron microscopy by fixation in 1.5% KMnO₄ for 20 min, followed by embedding in TAAB 812 resin (Marivac, Halifax, Nova Scotia, Canada). For immunoelectronmicroscopy, subcellular fractions were fixed in 3% (wt/vol) paraformaldehyde/0.2% (wt/vol) glutaraldehyde for 1 h at 4°C and embedded in LR Gold resin (Electron Microscopy Sciences, Fort Washington, PA). Immunodetection was performed with rabbit anti-SKL primary antibodies and goat anti–rabbit IgG secondary antibodies conjugated to 10 nm colloidal gold (Sigma Chemical Co., Mississauga, Ontario, Canada). Immunofluorescence microscopy was performed as described (Aitchison et al., 1992; Szilard et al., 1995).

**Epitope Tagging of Pex16p and Overexpression of the PEX16 Gene**

Various *PEX16* expression plasmids were constructed as follows. The *PEX16* gene, along with 965 and 16 bp of genomic DNA 5’ and 3’, respectively, to the ORF, was amplified by PCR of p16N1 (Fig. 2A) using primers 222 (5’) and 224 (3’) (Table II) and inserted as a blunt-ended fragment into the Smal site of pGEM7Zf(-) (Promega Corp., Madison, WI). The insert was excised with HindIII and ligated into a shuttle vector pTEC with a unique EcoRI cloning site between the promoter and terminator regions. The ORF of the *PEX16* gene was also amplified by PCR of p16N1 using oligonucleotides 249 (5’) and 224 (3’) (Table II) and cloned into pGEM7Zf(-) as a blunt-ended fragment. The insert was excised by digestion with EcoRI and ligated into the EcoRI site of pTEC. The *PEX16* ORF flanked by the thiolase gene promoter and terminator regions was excised by digestion with BglII and cloned into a shuttle vector to make the plasmid p16TH.

*PEX16* constructs encoding Pex16p-HA or flanked by the thiolase promoter/terminator regions were integrated into the *Y. lipolytica* genome by gene conversion (Rothstein, 1991) to control for gene copy number and to ensure stability of recombinant gene expression. The insert of p16HA or p16TH, along with the *URA3* gene, was cloned into the plasmid pSP73 (Promega). The *HA*-tagged version of the *PEX16* gene was integrated into the *pex16-1* site by cutting upstream of the *PEX16* gene. The thiolase gene promoter/terminator version of the *PEX16* gene was integrated into the endogenous *ura3*-302 site by cutting within the *URA3* gene of the integration construct. Linearized DNA was introduced into the *pex16-1* mutant by electroporation, and *Ura*+ transformants were selected. Correct integration was determined by Southern blot analysis.

**Analytical Procedures**

Enzymatic activities of the peroxisomal marker catalase (Luck, 1963) and the mitochondrial markers cytochrome c oxidase (Douma et al., 1985) and fumarase (Tolbert, 1974) were measured by established procedures. Protein concentration was measured as described by Bradford (1976) using ovalbumin as a standard. Total nucleic acid was isolated by glass bead lysis and phenol extraction, as previously described (Eitzen et al., 1995). Northern blot and Southern blot analyses were performed as described by Ausabel et al. (1989). Densitometry was performed using a laser densitometer (Ultrascan XL; LKB Instruments, Bromma, Sweden) (Szilard et al., 1995).

**Results**

**Isolation of the PEX16 Gene**

The *pex16-1* mutant was isolated from randomly mutated *Y. lipolytica* cells by screening for the inability to use oleic acid as a sole carbon source (Fig. 1). Several biochemical and morphological criteria (data presented below) were used to determine that this strain was affected in peroxisome assembly (*pex*, or formerly *pay*, phenotype) (Nuttley et al., 1993). The *PEX16* gene was isolated from a library of *Y. lipolytica* genomic DNA by functional complementation of the *pex16-1* strain. Approximately 10⁶ leucine prototroph (Leu⁺) transformants were screened.

---

**Table II. Oligonucleotides**

| Oligonucleotide | Sequence |
|-----------------|----------|
| THpr5’ | 5’-TCA TAA GCT TTG AGA ACC CCG AAG A-3’ |
| THpr3’ | 5’-GCT TAA GCT TCC AAT CAT CAA TCG CTT AGA-3’ |
| THtr5 | 5’-TTA AGC TTA AAG ATC TGC GGT GAA GTA GTA CCG AT-3’ |
| THtr3 | 5’-GAG ATC TAA CCT AGC GG-3’ |
| TTT | 5’-CAT CCG GTA ATT CAC ATG CAA G-3’ |
| 7-HA | 5’-GAT CCG CTA GGC ATG TAC CCA TAC GAC GTC CCA GAC |

**Peptide Tags**

| Peptide Tag | Sequence |
|-------------|----------|
| HA | GC GAT CCG TAG ATG AGT ATG CTC CAG GGT CTG |
| Y | Y A A M Y P Y D V P D Y |
| GCT GCC ATG GGT AGG GAA TAG AAG AAG AAG GATC T-3’ |

**Gene and Promoter Regions**

| Region | Sequence |
|--------|----------|
| p16TH | 5’-GAT CCG CTA GGC ATG TAC CCA TAC GAC GTC CCA GAC |
| 7-HA | 5’-GAT CCG CTA GGC ATG TAC CCA TAC GAC GTC CCA GAC |

**Gene Constructs**

| Gene Construct | Sequence |
|----------------|----------|
| p16TH | 5’-GAT CCG CTA GGC ATG TAC CCA TAC GAC GTC CCA GAC |
| pEX16 | 5’-GAT CCG CTA GGC ATG TAC CCA TAC GAC GTC CCA GAC |

**Enzyme Activities**

| Enzyme | Activity |
|--------|----------|
| Catalase | 2 U/mL |
| Cytochrome c oxidase | 1.5 nkat/mg |
| Fumarase | 0.2 U/mg |

**Isolation and Screening**

| Isolation Method | Screening Method |
|------------------|------------------|
| Southern blot | Northern blot |

**Media**

| Media | Composition |
|-------|-------------|
| YEPD | Yeast extract, peptone, dextrose |
| YEPG | Yeast extract, peptone, glycerol |

**Cloning Vectors**

| Vector | Features |
|--------|----------|
| pTEC | Unique EcoRI cloning site |
| pSP73 | Unique EcoRI cloning site |
| pGEM7Zf(-) | Unique EcoRI cloning site |

**Restriction Enzymes**

| Enzyme | Recognition Site |
|--------|------------------|
| BglII | 5’-GAG ATC TAA CCT AGC GG-3’ |
| EcoRI | 5’-GCT TAA GCT TCC AAT CAT CAA TCG CTT AGA-3’ |
| HindIII | 5’-TCA TAA GCT TTG AGA ACC CCG AAG A-3’ |

**Protein Expression**

| Expression System | Protein |
|-------------------|---------|
| PEX16 | Pex16p-HA |
| PEX16 promoter | Pex16p |

**Integration**

| Integration Site | Integration Strategy |
|------------------|----------------------|
| pSP73 | Gene conversion (Rothstein, 1991) |
| pGEM7Zf(-) | Gene conversion (Rothstein, 1991) |

**Immunoblotting**

| Antibody | Immunoreactive Band |
|----------|---------------------|
| Rabbit Anti-SKL | 50 kDa |
| Goat Anti–Rabbit IgG | 50 kDa |

**Immunofluorescence**

| Primary Antibody | Secondary Antibody |
|------------------|--------------------|
| Rabbit Anti-SKL | Goat Anti–Rabbit IgG |

**Electron Microscopy**

| Preparation Method | Observation |
|--------------------|-------------|
| Tolbert (1974) | Mitochondria |
| Luck (1963) | Peroxisomes |

**Densitometry**

| Method | Analysis Type |
|--------|---------------|
| Ultrascan XL | Semi-quantitative analysis |
| LKB Instruments | Quantitative analysis |

**Phenotypic Analysis**

| Phenotype | Description |
|----------|-------------|
| pex16-1 | Unable to use oleic acid as sole carbon source |
| HA-tagged *PEX16* | Increased expression of *PEX16* gene |

---

Eitzen et al. Peroxisome Biogenesis in Yarrowia lipolytica 1267
Two strains recovered growth on oleic acid (ole\(^{-}\)). Total DNA was isolated from these strains, and the complementing plasmids were recovered by transformation of E. coli. The recovered plasmids, p16N1 and p16C2, were mapped by restriction endonuclease digestion and were found to share a 5.5-kbp region (Fig. 2A). Subfragments of this region were cloned, and the minimum complementing subfragment was localized to a 2.1-kbp HindIII/ClaI restriction fragment contained within plasmid p16HC2 (Fig. 2A) in the transformed strain P16TR (Fig. 1).

Sequencing of the 2.1-kbp insert of p16HC2 revealed an ORF encoding a protein of 391 amino acids, Pex16p, with a predicted molecular weight of 44,479 (Fig. 2B). Hydrophathy analysis predicted the presence of several hydrophobic segments capable of membrane interaction (Fig. 2C). Pex16p was predicted to contain one membrane-spanning \(\alpha\)-helix and three membrane-associated helices (Fig. 2B). A search of protein data bases using the GENINFO(R) BLAST Network Service (Blaster) of the National Center for Biotechnology Information revealed no significant homology between Pex16p and any other known protein.

The putative PEX16 gene was disrupted by targeted integration of the \(Y.\) lipolytica \(LEU2\) gene to make the strains P16KO-8A and P16KO-8B; the diploid strains D16-E1x8B, D16-8Ax22, D16-P16x8B, and D16-P16x22; the strain pex16-1HA synthesizing Pex16p-HA; and the strain pex16-TH expressing the PEX16 gene from the thiolase promoter. The wild-type strain 22301-3 was not supplemented for its auxotrophic requirements. Growth on YNO requires at least one copy of the intact PEX16 gene.

Figure 1. Growth of various \(Y.\) lipolytica strains on oleic-acid-containing medium. The strains listed in Table I were grown for 3 d on YNO agar. The appearance of the original pex16-1 mutant is compared to that of the wild-type strain E122; the complemented strain P16TR; the gene disruption strains P16KO-8A and P16KO-8B; the diploid strains D16-E1x8B, D16-8Ax22, D16-P16x8B, and D16-P16x22; the strain pex16-1HA synthesizing Pex16p-HA; and the strain pex16-TH expressing the PEX16 gene from the thiolase promoter. The wild-type strain 22301-3 was not supplemented for its auxotrophic requirements. Growth on YNO requires at least one copy of the intact PEX16 gene.

Figure 2. Cloning and analysis of the PEX16 gene. (A) Complementing activity of inserts, restriction map analysis, and targeted gene disruption strategy for the PEX16 gene. (Solid lines) \(Y.\) lipolytica genomic DNA; (open boxes) vector DNA. The ORFs of the PEX16 and LEU2 genes are indicated by the wide arrows. The (+) symbol denotes the ability and the (−) symbol the inability of an insert to confer growth on oleic acid to \(pex16-1\). (B) Nucleotide sequence of the PEX16 gene and deduced amino acid sequence of Pex16p. (Underlined residues) Predicted membrane-associated \(\alpha\)-helices; (doubly underlined residues) predicted transmembrane \(\alpha\)-helix. These sequence data are available from EMBL/GenBank/DDJB under accession number U75433. (C) Hydrophathy profile of Pex16p calculated according to Kyte and Doolittle (1982) with a window size of 15 amino acids. Four hydrophobic domains predicted to interact with membranes are underlined.
D16-E1x8B from the mating of wild-type strain E122 to strain P16KO-8B (Table I), could grow on oleic acid–containing medium (Fig. 1), showing the recessive nature of the pex16-1 mutation. The diploid strain D16-P16x8B made by mating the original pex16-1 mutant strain to strain P16KO-8B (Table I) could not grow on oleic acid-containing medium (Fig. 1). Accordingly, the ability to utilize oleic acid as the sole carbon source required at least one intact copy of the PEX16 gene, thereby confirming that the authentic PEX16 gene had been cloned.

pex16 Cells Lack Normal Peroxisomes but Show Evidence of Peroxisomal Structures

Normal peroxisomes of *Y. lipolytica* appear as round vesicular structures, 0.2–0.5 μm in diameter, with a granular electron-dense core and a single unit membrane (Fig. 3A). The original mutant strain pex16-1 (Fig. 3B) and the disruption strain P16KO-8A (Fig. 3D) grown in oleic acid–containing medium lacked normal peroxisomes. However, these strains did show clusters of small (40–50 nm diam) vesicles (Fig. 3, B and D, arrows, and B, inset) and occasionally larger (100–150 nm diam) vesicular structures (Fig. 3, B and D, arrowheads, and D, inset). Morphologically similar structures could be seen in the wild-type strain (compare Fig. 3A); however, extensive clustering of the smaller vesicles was rarely, if ever, observed in these cells. The transformed strain P16TR had the appearance of the wild-type strain and showed normal peroxisome morphology (Fig. 3C).

Although pex16 mutants lacked normal peroxisomes, subcellular fractionation provided evidence of peroxisomal structures in these strains. Wild-type and mutant strains were grown in oleic acid–containing medium to proliferate peroxisomes and then were fractionated into a 20KgP fraction enriched for peroxisomes and mitochondria and a 20KgS fraction, as described in Materials and Methods. In the wild-type strain E122, >75% of each peroxisomal protein was localized to the 20KgP fraction (Fig. 4). In contrast, in the original mutant strain pex16-1 and in the disruption strain P16KO-8A, several peroxisomal proteins were almost completely mislocalized to the 20KgS fraction.

**Figure 3.** Ultrastructure of wild-type and pex16 mutant strains. The E122 (A), pex16-1 (B), P16TR (C), and P16KO-8A (D) strains were grown in YEPD medium to an OD600 of ~1.5, transferred to YPBO medium at a dilution of 1:4, and grown for an additional 8 h in YPBO. Cells were fixed in K2MnO4 and processed for electron microscopy. P, peroxisome; M, mitochondrion; N, nucleus; V, vacuole. (Arrows) Vesicular structures of 40–50 nm diam. (Arrowheads) Vesicular structures 100–150 nm diam. Inset in B shows a cluster of vesicles of 40–50 nm diam. Inset in D shows vesicles of 100–150 nm. Bars: (A–D) 0.5 μm; (inset) 0.1 μm.
fraction, while others were only partially mislocalized (Fig. 4). Isocitrate lyase, thiolase, and catalase were found almost exclusively (85.5–99.4%) in the 20KgS fraction of the mutant strains. Malate synthase and the peroxisomal membrane proteins Pex2p (Eitzen et al., 1996) and Pex5p (Szilard et al., 1995) were partially mislocalized (38–79%) to the 20KgS, while acyl-CoA oxidase and a 62-kD anti-SKL reactive polypeptide were localized to the 20KgP fraction at levels comparable to those for the wild-type strain. The mitochondrial markers cytochrome c oxidase and fumarase were preferentially localized to the 20KgP fraction in both the wild-type and mutant strains (Fig. 4). Protease protection experiments showed that peroxisomal matrix proteins localized to the 20KgP fraction of the pex16-l mutant were resistant to digestion by external protease in the absence, but not in the presence, of detergent (Fig. 5), consistent with their localization within membrane-enclosed structures.

Immunofluorescence analysis of oleic acid–grown wild-type cells probed with anti-SKL antibodies showed a punctate pattern of staining characteristic of peroxisomes (Fig. 6 A). In contrast, pex16-l cells (Fig. 6 B) probed with the same antibodies showed either a more generalized pattern of staining or a punctate pattern suggestive of peroxi-
somal structures that were smaller and less fluorescent than wild-type peroxisomes (Fig. 6 A). In an attempt to gain some initial insight into the nature of these structures, the 20KgP fractions of the wild-type and mutant strains were subfractionated by isopycnic centrifugation on discontinuous sucrose density gradients (see Materials and Methods). Fractions were analyzed for density, protein content, catalase and fumarase activities (Fig. 7 A), and by immunoblotting with antibodies to peroxisomal matrix (anti-SKL reactive polypeptides, acyl-CoA oxidase, and thiolase) and membrane (Pex2p) proteins (Fig. 7 B). In the wild-type strain E122, peroxisomal proteins were localized primarily to fractions 3–5, peaking in fraction 4 at a density of 1.21 g/cm³, while mitochondria were well separated from peroxisomes in fractions 9–11, peaking at 1.18 g/cm³. In the pex16-1 and P16KO-8A mutant strains, two peaks of peroxisomal proteins were observed, a lesser peak at a density of 1.21 g/cm³ and a greater peak at a density of 1.16 g/cm³ (fractions 9–12). It is noteworthy that the more slowly migrating anti-SKL reactive polypeptide of 64 kD was not present in the immunoblots of the fractions of

Figure 5. Peroxisomal matrix proteins localized to the 20KgP fraction of the pex16-1 mutant are resistant to the action of external protease. The 20KgP fraction of mutant strain pex16-1 was isolated in the absence of protease inhibitors. 200 μg of protein of this fraction was incubated with 0, 2, 5, 10, and 25 μg of trypsin in the absence (−) or presence (+) of 0.5% (vol/vol) Triton X-100 on ice for 30 min. Reactions were terminated by addition of hot SDS-PAGE sample buffer and immediate boiling. Samples were analyzed by immunoblotting with anti–acyl-CoA oxidase (AOX), anti-SKL (SKL), and anti-thiolase (THI) antibodies.

Figure 6. Immuno-microscopic analysis of whole cells and subcellular fractions of the wild-type and pex16-1 mutant strains. The wild-type strain E122 (A) and the mutant strain pex16-1 (B) were grown for 8 h in YPBO, as described in the legend to Fig. 3. Cells were processed for immunofluorescence microscopy with rabbit anti-SKL antibodies and FITC-conjugated goat anti-rabbit IgG antibodies. (C–F) Subcellular fractions were isolated by isopycnic gradient analysis of 20KgP fractions of the wild-type E122 and the mutant pex16-1 strains as described in Materials and Methods and the legend to Fig. 7. Gradient fractions 4 and 11 of the wild-type strain (C and D, respectively) and of the pex16-1 strain (E and F, respectively) were fixed with paraformaldehyde/glutaraldehyde and processed for immuno-electronmicroscopy with anti-SKL antibodies. Peroxisomes in fraction 4 of the wild-type strain (C) and vesicular structures in both fraction 4 (E) and fraction 11 (F) of the pex16-1 strain are decorated with immunogold. Mitochondria are essentially undecorated. Bars: (A and B) 5 μm; (C, D, and F) 0.5 μm; (E) 0.25 μm.
the pex16 mutant strains (Fig. 7 B). This 64-kD polypeptide corresponds to isocitrate lyase (Eitzen, G.A., and R.A. Rachubinski, unpublished results), and as presented in Fig. 4, most isocitrate lyase was mislocalized to the 20KgS in the pex16 strains.

Gradient fractions were also analyzed by immuno-electronmicroscopy using anti-SKL antibodies (Fig. 6). Fraction 4 (1.21 g/cm³, the normal density of peroxisomes) from the wild-type strain showed mainly large, round electron-dense profiles characteristic of peroxisomes (Fig. 6 C). These profiles were decorated with gold particles. The occasional mitochondria present in this fraction were largely undecorated by gold particles (Fig. 6 C). Fraction 4 of the pex16-1 mutant showed predominantly round, electron-dense profiles decorated by gold particles (Fig. 6 E). These structures were smaller in diameter than wild-type peroxisomes. Wild-type fraction 11 (1.16 g/cm³) contained predominantly undecorated mitochondria (Fig. 6 E). Fraction 11 from the pex16-1 mutant strain contained mitochondria and, in addition, small electron-dense structures decorated with gold particles (Fig. 6 F).

**Pex16p Is a Peripheral Protein Preferentially Associated with the Matrix Face of the Peroxisomal Membrane**

Antibodies raised against a maltose-binding protein–Pex16p fusion protein recognized a polypeptide of ~43 kD in extracts of oleic acid-grown E122 cells but not of pex16-1 cells (Fig. 8 A). The molecular mass of this polypeptide is close to the predicted molecular mass of Pex16p, 44,479 D. No immunoreactive 43-kD polypeptide was seen in the lysate of the disruption strain P16KO-8A, but it was present in the lysate of the transformed strain P16TR (Fig. 8 A). Therefore, the antibodies specifically recognize Pex16p. Immunoblot analysis of subcellular fractions and peroxisomes purified from oleic acid–grown wild-type cells, with anti-Pex16p antibodies, showed Pex16p to be localized to peroxisomes (Fig. 8 B, lane PX). Pex16p was absent from the 20KgS fraction (Fig. 8 B, lane S). Lysis of peroxisomes with Ti8 buffer, followed by high speed centrifugation, showed Pex16p to be localized exclusively to the pellet containing membrane proteins (Fig. 8 C, middle and bottom, lanes PTi8) and not to the supernatant containing soluble matrix proteins (Fig. 8 C, top, lane STi8). Increasing the stringency of the extraction buffer by the addition of 0.5 M KCl to the Ti8 buffer (TS buffer) led to partial extraction of Pex16p (Fig. 8 C, bottom, compare lane PTS to lane STS) but not of the integral membrane protein Pex2p (Fig. 8 C, bottom, compare lane PPTS to lane SPTS) from the membrane pellet. The more quickly migrating species of Pex16p in lane SPTS was probably due to nonspecific proteolysis. Treatment of peroxisomes with 0.1 M Na₂CO₃, pH 11.5, led to the complete extraction of Pex16p but not of Pex2p from the membrane pellet (Fig. 8 C, middle and bottom, respectively; compare lanes P CO to lanes S CO).
in the absence, but not in the presence, of the detergent Pex16p was protected from the action of external protease subjected to protease protection analysis (Fig. 8). The type strain grown in oleic acid–containing medium was mal membrane, the 20KgP fraction isolated from the wild-type strain grown for 8 h in YPBO medium, as described in the legend to Fig. 3, and then subjected to subcellular fractionation to yield PNS, 20KgS (S), and 20KgP (P) fractions. Equal portions of the fractions (0.1% of the total volume) were probed with 12CA5 antibodies to localize Pex16p-HA (arrowhead). Immunoblot analysis of the 20KgP of the pex16-1 strain fractionated by isopycnic density gradient centrifugation. Equal volumes of the fractions were probed with anti-SKL antibodies, as shown by its detection in wild-type strain fractionated by isopycnic density gradient centrifugation.

Therefore, Pex16p shows the characteristics of a protein that is preferentially associated with the matrix face of the peroxisomal membrane. Anti-Pex16p antibodies recognize a polypeptide of ~43 kD in lysates of the wild-type strain E122 and the transformed strain P16TR but not in lysates of the original mutant strain pex16-1 or the disruption strain P16KO-8A. The numbers at left indicate the migrations of molecular mass standards (in kilodaltons). Immunoblot analysis of subcellular fractions PNS, 20KgS (S), and 20KgP (P), and of whole peroxisomes (PX, 30 μg of protein) of the wild-type strain grown as in A and probed with anti-Pex16p antibodies. Equal fractions (0.1% of the total volume) of the PNS, 20KgS, and 20KgP were loaded. (C) Immunoblot analysis of peroxisomes separated into pellet (P) and supernatant (S) fractions by treatment with Ti8, TS, or sodium carbonate buffer. The upper blot was probed with anti-SKL antibodies to detect peroxisomal matrix proteins. The middle blot was probed with anti-Pex16p antibodies. The lower blot was probed with antibodies to the peroxisomal integral membrane protein Pex2p. (D) Protease protection analysis of anti-SKL reactive polypeptides and Pex16p. The 20KgP fraction (200 μg of protein) isolated in the absence of protease inhibitors from the wild-type strain E122 was incubated with the protease trypsin in the absence (−) or presence (+) of 0.5% (vol/vol) Triton X-100. Peroxisomal matrix proteins recognized by anti-SKL antibodies behaved similarly to Pex16p in this analysis (Fig. 8 D). These results indicate that Pex16p is associated preferentially with the matrix face of the peroxisomal membrane.

![Figure 8](Image)

Figure 8. Pex16p is a peripheral protein preferentially associated with the matrix face of the peroxisomal membrane. (A) Immunoblot analysis of whole cell extracts (50 μg of protein) of strains grown for 8 h in YPBO medium, as described in the legend to Fig. 3. Anti-Pex16p antibodies recognize a polypeptide of ~43 kD in lysates of the wild-type strain E122 and the transformed strain P16TR but not in lysates of the original mutant strain pex16-1 or the disruption strain P16KO-8A. The numbers at left indicate the migrations of molecular mass standards (in kilodaltons). (B) Immunoblot analysis of subcellular fractions PNS, 20KgS (S), and 20KgP (P), and of whole peroxisomes (PX, 30 μg of protein) of the wild-type strain grown as in A and probed with anti-Pex16p antibodies. Equal fractions (0.1% of the total volume) of the PNS, 20KgS, and 20KgP were loaded. (C) Immunoblot analysis of peroxisomes separated into pellet (P) and supernatant (S) fractions by treatment with Ti8, TS, or sodium carbonate buffer. The upper blot was probed with anti-SKL antibodies to detect peroxisomal matrix proteins. The middle blot was probed with anti-Pex16p antibodies. The lower blot was probed with antibodies to the peroxisomal integral membrane protein Pex2p. (D) Protease protection analysis of anti-SKL reactive polypeptides and Pex16p. The 20KgP fraction (200 μg of protein) isolated in the absence of protease inhibitors from the wild-type strain E122 was incubated with the protease trypsin in the absence (−) or presence (+) of 0.5% (vol/vol) Triton X-100. Peroxisomal matrix proteins recognized by anti-SKL antibodies behaved similarly to Pex16p in this analysis (Fig. 8 D). These results indicate that Pex16p is associated preferentially with the matrix face of the peroxisomal membrane.

Carboxyl Terminus of Pex16p Is Not Essential for Its Function or Targeting

Pex16p has at its carboxyl terminus the tripeptide sequence, Ser-Thr-Leu (Fig. 2 B). We investigated whether this tripeptide was a variant of the prototypical PTS1 sequence, Ser-Lys-Leu and was necessary for targeting Pex16p to peroxisomes. The plasmid p16HA encodes a modified Pex16p (Pex16p-HA) that has two copies of the HA epitope tag appended to its carboxyl terminus and ends in the tripeptide, Lys-Gly-Glu, which does not resemble the PTS1 consensus motif. Transformation of strains pex16-1 and P16KO-8A with p16HA reestablished growth on oleic acid medium (data not shown). Therefore, Pex16p-HA was capable of functional complementation of the pex16 strains.

To ensure that functional complementation of the pex16 strains by the plasmid p16HA was not due to overexpression of the modified PEX16 gene, we constructed the strain pex16-1HA (Table I), in the pex16-1 background, that harbors a single copy of the modified PEX16 gene under the control of the PEX16 promoter and coding for Pex16p-HA. This strain recovered growth on oleic acid medium (Fig. 1). Immunoblot analysis of subcellular fractions prepared from the pex16-1HA strain grown in oleic acid–containing medium showed localization of Pex16p-HA preferentially to the 20KgP fraction (Fig. 9 A, arrowhead, lane P) and to purified peroxisomes (Fig. 9 B). A polypeptide with reduced electrophoretic mobility relative to Pex16p-HA was nonspecifically recognized by 12CA5 antibodies, as shown by its detection in wild-type E122 cells not synthesizing Pex16p-HA (Fig. 9 A, right). Our results show that the amino acids Ser-Thr-Leu at the car-
The boxyl terminus of Pex16p are not essential for its function or targeting to peroxisomes.

**Oversynthesis of Pex16p Results in Fewer but Enlarged Peroxisomes**

Immunofluorescence analysis of oleic acid–grown cells probed with antibodies directed against peroxisomal matrix proteins showed a punctate pattern characteristic of peroxisomes in wild-type cells (Fig. 10 A, and compare Fig. 6 A) but not in pex16 cells (compare Fig. 6 B). Expression of PEX16 in the transformed strain P16TR restored the characteristic punctate pattern of staining of peroxisomes (Fig. 10 B). To examine the effects of oversynthesis of Pex16p, the strain pex16-TH (Table I) expressing the PEX16 gene from the thiolase gene promoter was grown in oleic acid–containing medium. Immunoblot analysis showed that Pex16p synthesis was increased 10-fold after 8 h of growth in oleic acid–containing medium in the strain pex16-TH as compared with the wild-type strain E122 (Fig. 11, Pex16p), while the levels of expression of peroxisomal matrix proteins in the two strains remained un-
changed (Fig. 11, SKL). It is interesting to note that Pex16p was synthesized by wild-type cells grown in glucose-containing medium (time = 0 h) and that the level of Pex16p only modestly increased during growth in oleic acid-containing medium (Fig. 11).

Immunofluorescence microscopy of the pex16-TH strain showed the effects of oversynthesis of Pex16p. After 8 h of growth in oleic acid-containing medium, cells of the pex16-TH strain contained 1–5 enlarged peroxisomes (Fig. 10, C and D) instead of the 25–50 peroxisomes normally seen in wild-type cells grown under the same conditions (Figs. 6 A and 10 A). Since the ability to grow in oleic acid–containing medium was restored in the strain pex16-TH (Fig. 1), these enlarged peroxisomes are functional.

Electron microscopic analysis showed that pex16-TH cells contained peroxisomes with an average diameter of \(0.87 \pm 0.14 \mu m\), while wild-type cells contained peroxisomes with an average diameter of \(0.36 \pm 0.10 \mu m\). The increased size of peroxisomes in the pex16-TH strain apparently does not affect their inheritance, since cell sections routinely showed large peroxisomes in both halves of a dividing cell (Fig. 12 A).

Peroxisomal proteins were preferentially localized to the 20KgP isolated from the pex16-TH strain at levels approaching those of oversynthesis of Pex16p from the wild-type strain (Fig. 12 B). The reduction in levels of peroxisomal proteins in the 20KgP fraction from the pex16-TH strain may be due to an increased fragility of the enlarged peroxisomes vis-à-vis wild-type peroxisomes during subcellular fractionation and/or to a reduced overall number of protein import sites in the enlarged peroxisomes. The enlarged peroxisomes of the pex16-TH strain could be isolated by isopycnic centrifugation of the 20KgP on a discontinuous sucrose gradient (Fig. 12 C). These enlarged peroxisomes peaked at a density of \(1.21 g/cm^3\), the same as wild-type peroxisomes (compare Fig. 7 A), and contained catalase (Fig. 12 C) and all other peroxisomal matrix and membrane proteins tested (data not shown). Mitochondria from the pex16-TH strain peaked at a density of \(1.18 g/cm^3\), like those of the wild-type strain (compare Fig. 7 A).

**Discussion**

Here we report the following: the isolation and characterization of *Y. lipolytica* pex16 mutant strains, the cloning and sequencing of the *PEX16* gene, the identification and characterization of the peroxin Pex16p, and an analysis of the effects of overexpression of the *PEX16* gene.

**Absence of Functional Peroxisomes but Evidence of Peroxisomal Structures in pex16 Mutant Strains**

pex16 mutant strains cannot assemble functional peroxisomes. They are unable to grow using oleic acid as the sole carbon source, and under conditions of peroxisome induction, they fail to proliferate morphologically normal peroxisomes. However, subcellular fractionation showed that pex16 strains import a subset of peroxisomal proteins at, or near, wild-type levels, suggesting that they are not generalized mutants of peroxisomal protein import. While isocitrate lyase, thiolase, and catalase are completely mislocalized to a 20,000 \(g_{max}\) supernatant fraction (20KgS) in pex16 mutants, other peroxisomal matrix (acyl-CoA oxidase, a 62-kD anti-SKL reactive polypeptide, malate synthase) and membrane (Pex2p and Pex5p) proteins are localized to an organellar fraction pelletable at 20,000 \(g_{max}\) (20KgP).

Peroxisomal proteins pelletable to the 20KgP are contained in elements that equilibrate at densities of 1.21 and 1.16 g/cm³. Preliminary immunocytochemical analysis sug-
gests that both of these fractions contain vesicular structures of varying diameters that were labeled by anti-SKL antibodies and immunogold (compare Fig. 6, E and F). These or similar vesicular structures were visible in electron micrographs of whole cells of the pex16 mutant strains and, to a lesser extent, of the wild-type strain (compare Fig. 6, B and A, respectively). In contrast, immunoelectronmicrographs of fractions from wild-type cells showed a preponderance of decorated profiles typical of peroxi-

![Image](https://example.com/image1)

**Figure 12.** Characterization of the enlarged peroxisomes of the pex16-TH strain. (A) Electron micrograph of a dividing cell of strain pex16-TH. The cell appears normal except for enlarged peroxisomes. Enlarged peroxisomes are present in both halves of the dividing cell. (B) The PEX16 overexpression strain, pex16-TH, was grown for 8 h in YPBO medium and subjected to subcellular fractionation to yield 20Kgs and 20Kgp fractions. The distribution of the enzymatic activities of catalase (CAT) and fumarase (FUM) and of the immunosignals of acyl-CoA oxidase (AOX), isocitrate lyase (ICL), a 62-kD anti-SKL reactive polypeptide (SKL), thiolase (THI), and Pex2p to the 20Kgs and 20Kgp fractions are reported as the percent recovery of the respective total enzymatic activity or immuno
signal in the PNS fraction. Immunoblots were quantitated by densitometry. Values reported are the means ±SD of two independent experiments, each analyzed in duplicate. (C) Isopycnic density gradient analysis of the 20Kgp from the strain pex16-TH grown for 8 h in YPBO medium. Distribution of protein (—), catalase (solid bars), fumarase (open bars), and density (solid line). Values reported are the means of two independent experiments, each analyzed in duplicate. P, peroxisome; M, mitochondrion; N, nucleus. Bar, 0.5 μm.
Carboxyl-terminal Tripeptide Ser-Thr-Leu of Pex16p Is Not Essential for Its Targeting to Peroxisomes

Pex16p has at its carboxyl terminus the tripeptide Ser-Thr-Leu. This sequence is very similar to the consensus sequence of PTS1 motifs, (Ser/Ala/Cys)(Lys/Arg/His)(Leu/Met). We therefore investigated whether this tripeptide motif is required for the targeting of Pex16p to peroxisomes. Elimination of the tripeptide by addition of the HA epitope to the carboxyl terminus of Pex16p does not prevent its targeting to peroxisomes (compare Fig. 9). Therefore, the carboxyl-terminal tripeptide of Pex16p is not required for its targeting to peroxisomes.

Peroxisomes are able to import oligomeric and folded proteins (Glover et al., 1994; McNew and Goodman, 1994, 1996; Walton et al., 1995). The subunit composition of Pex16p is unknown. However, the gene encoding Pex16p-HA was expressed in the P16KO-8A background, in which the ORF of the nuclear PEX16 gene is deleted. Accordingly, targeting of Pex16p-HA to peroxisomes cannot be the result of mimerization with a wild-type Pex16p having its carboxyl-terminal tripeptide intact. However, we cannot rule out the possibility that Pex16p-HA could gain access to the peroxisome by heterodimerizing with a different peroxisomal protein. Nevertheless, such a scenario would still be consistent with the carboxyl-terminal tripeptide of Pex16p not being necessary for its targeting to peroxisomes.

What Could be the Function of Pex16p?

What role might Pex16p play in peroxisome biogenesis? Our results indicate that Pex16p is a component of the peroxisome assembly machinery and suggest that Pex16p may in fact have more than one function. Pex16p may facilitate the import of some peroxisomal proteins, notably of catalase, thiolase, and isocitrate lyase. These enzymes are not localized to peroxisomal structures that pellet to the 20kG pellet and therefore do not contribute to the 20kG pellet in frozen samples. However, the levels of these enzymes in the enlarged peroxisomes of the PEX16 overexpression strain approach the levels found in wild-type peroxisomes (compare Figs. 10 and 12). Whether facilitation of import of this subset of peroxisomal proteins can be attributed directly to the functioning of Pex16p cannot be answered at this time.

Our results also suggest that Pex16p may be involved in peroxisome proliferation. Interestingly, overexpression of the PEX16 gene in oleic acid–grown Y. lipolytica results in a reduced number of enlarged peroxisomes as compared to wild-type cells. Other peroxins have been implicated in peroxisomal proliferation or fission, although their actions contrast with those of Pex16p. Overexpression of Pex3p and Pex10p in H. polymorpha (Baerends et al., 1996; Tan et al., 1995) and Pex11p in S. cerevisiae and C. boidinii (Marshall et al., 1995; Sakai et al., 1995) leads to a proliferation of normal peroxisomes, while elimination of Pex11p in these yeast species leads to a small number of enlarged peroxisomes (Erdmann and Blobel, 1995; Marshall et al., 1995; Sakai et al., 1995). Whether Pex16p and these peroxins act themselves as positive and negative factors of peroxisome proliferation cannot be answered at this time. It will be interesting to determine whether Pex16p interacts with these peroxins and to identify novel partners of Pex16p so as to provide insight into the molecular machinery controlling peroxisome structure, growth, and proliferation.

We thank Honey Chan for assistance with electron microscopy.

G.A. Eitzen is the recipient of a Studentship from the Alberta Heritage Foundation for Medical Research. R.K. Szilard is the recipient of a Studentship from the Medical Research Council (MRC) of Canada. R.A. Rachubinski is an MRC Scientist and an International Research Scholar of the Howard Hughes Medical Institute. This work was supported by a grant from the MRC to R.A. Rachubinski.

Received for publication 6 November 1996 and in revised form 17 March 1997.

References

Aitchison, J.D., W.W. Murray, and R.A. Rachubinski. 1991. The carboxyl-terminal tripeptide Ala-Lys-Ile is essential for targeting Candida tropicalis tri-functional enzyme to yeast peroxisomes. J. Biol. Chem. 266:23197–23203.

Aitchison, J.D., R.K. Szilard, W.M. Nuttley, and R.A. Rachubinski. 1992. Antibodies directed against a yeast carboxyl-terminal peroxisomal targeting signal specifically recognize peroxisomal proteins from various yeasts. Yeast. 8: 721–734.

Anubel, F.J., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1989. In Current Protocols in Molecular Biology. Green Publishing Associates, New York. 3:1–3.17, 13.11–13.12.

Baerends, R.J.S., S.W. Rasmussen, R.E. Hilbrands, M. van der Heide, K.N. Faber, P.T.W. Reuvekamp, J.A.K.W. Kiel, J.M. Cregg, I.J. van der Klei, and M. Veenhuis. 1996. The Hansenaure polyomorpha PEX9 gene encodes a peroxisomal membrane protein essential for peroxisome assembly and integrity. J. Biol. Chem. 271:8887–8894.

Berninger, G., R. Schmidtchen, G. Casel, A. Knorr, K. Rautenstrauss, W.-H. Kunau, and E. Schweizer. 1993. Structure and metabolic control of the Yarrowia lipolytica peroxisomal 3-oxoacyl-CoA thiolase gene. Eur. J. Biochem. 216:607–613.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye bind-

De Duve, C., and P. Baudhuin. 1966. Peroxosomes (microbodies and related particles). Physiol. Rev. 46:323–357.

Distel, B., R. Erdmann, S.J. Gould, G. Blobel, D.I. Crane, J.M. Cregg, G. Dodt, Y. Fujiaki, J.M. Goodman, W.W. Just, et al. 1996. A unified nomenclature for peroxisomal biogenesis. J. Cell Biol. 135:3–1.

Douma, A.C., M. Veenhuis, W. de Koning, M. Evers, and W. Harder. 1985. Di-hydroxyacetone synthase is localized in the peroxisomal matrix of methanol-grown Hansenaure polyomorpha. Arch. Microbiol. 143:237–243.

Dyer, J.M., J.A. McNew, and J.M. Goodman. 1996. The sorting sequence of the peroxisomal integral membrane protein PMP47 is contained within a short hydrophilic loop. J. Cell Biol. 133:269–280.

Eitzen, G.A., J.D. Aitchison, R.K. Szilard, M. Veenhuis, W.M. Nuttley, and R.A. Rachubinski. 1995. The Yarrowia lipolytica gene PAY2 encodes a 42-kDa peroxisomal integral membrane protein essential for matrix protein import and peroxisome enlargement but not for peroxisome membrane proliferation. J. Biol. Chem. 270:1429–1436.

Eitzen, G.A., V.J. Titorenko, J.J. Smith, M. Veenhuis, R.K. Szilard, and R.A. Rachubinski. 1996. The Yarrowia lipolytica gene a double-stranded DNA fragment of the peroxisomal membrane encodes a peroxisomal integral membrane protein homologous to the mammalian peroxisome assembly factor PAF-1. J. Biol. Chem. 271:20300–20306.

Erdmann, R., and G. Blobel. 1995. Giant peroxisomes in oleic acid-induced Saccharomyces cerevisiae lacking the peroxisomal membrane protein Pmp27. J. Cell Biol. 128:509–523.

Glover, J.R., D.W. Andrews, and R.A. Rachubinski. 1994. Saccharomyces cerevisiae peroxisomal thiolase is imported as a dimer. Proc. Natl. Acad. Sci. USA. 91:10541–10545.

Häusler, T., Y.-D. Stierhof, E. Wirtz, and C. Clayton. 1996. Import of a DHFR hybrid protein into glycosomes in vivo is not inhibited by the folate analogue aminopterin. J. Cell Biol. 132:311–324.

Kyte, J., and R.F. Doolittle. 1982. A simple method for displaying the hydrophobic character of a protein. J. Mol. Biol. 135:31–4.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.

Lazarow, P.B., and C. de Duve. 1976. A fatty acyl-CoA oxidizing system in rat liver peroxisomes: enhanced by clofibrate, a hypolipidemic drug. Proc. Natl. Acad. Sci. USA. 73:2043–2046.

Lazarow, P.B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. Annu. Rev. Cell
Lazarow, P.B., and H.W. Moser. 1994. Disorders of peroxisome biogenesis. In The Metabolic Basis of Inherited Disease. 7th Ed. C.R. Scriver, A.L. Beaudet, W.S. Sly, and A.D. Valle, editors. McGraw-Hill, New York. 2287–2324.

Luck, H. 1963. Catalase. In Methods of Enzymatic Analysis. H.-U. Bergmeyer, editor. Academic Press, New York. 885–888.

Marshall, P.A., Y.I. Krimkevich, R.H. Lark, J.M. Dyer, M. Veenhuis, and J.M. Goodman. 1995. Pmp27 promotes peroxisomal proliferation. J. Cell Biol. 129:345–355.

McNew, J.A., and J.M. Goodman. 1994. An oligomeric protein is imported into peroxisomes in vivo. J. Cell Biol. 127:1245–1257.

McNew, J.A., and J.M. Goodman. 1996. The targeting and assembly of peroxisomal proteins: some old rules do not apply. Trends Biochem. Sci. 21:54–58.

Nuttley, W.M., A.M. Brade, C. Gaillardin, G.A. Eitzen, J.R. Glover, J.D. Aitchison, and R.A. Rachubinski. 1993. Rapid identification and characterization of peroxisomal assembly mutants in Yarrowia lipolytica. Yeast. 9:507–517.

Rachubinski, R.A., and S. Subramani. 1995. How proteins penetrate peroxisomes. Cell. 83:525–528.

Rothstein, R. 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. Methods Enzymol. 194:281–301.

Sakai, Y., P.A. Marshall, A. Saiganji, K. Takabe, H. Saiki, N. Kato, and J.M. Goodman. 1995. The Candida boidinii peroxisomal membrane protein Pmp30 has a role in peroxisomal proliferation and is functionally homologous to Pmp27 from Saccharomyces cerevisiae. J. Bacteriol. 177:6773–6781.

Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. Annu. Rev. Cell Biol. 9:445–478.

Subramani, S. 1996. Protein translocation into peroxisomes. J. Biol. Chem. 271:32483–32486.

Szilard, R.K., V.I. Titorenko, M. Veenhuis, and R.A. Rachubinski. 1995. Pay2p of the yeast Yarrowia lipolytica is an intraperoxisomal component of the matrix protein translocation machinery. J. Cell Biol. 131:1453–1469.

Tan, X., H.R. Waterham, M. Veenhuis, and J.M. Cregg. 1995. The Hansenula polymorpha PER8 gene encodes a novel peroxisomal integral membrane protein involved in proliferation. J. Cell Biol. 128:307–319.

Titorenko, V.I., G.A. Eitzen, and R.A. Rachubinski. 1996. Mutations in the PAY5 gene of the yeast Yarrowia lipolytica cause the accumulation of multiple subpopulations of peroxisomes. J. Biol. Chem. 271:20307–20314.

Tolbert, N.E. 1974. Isolation of subcellular organelles of metabolism on isopycnic sucrose gradients. Methods Enzymol. 31:734–746.

Veenhuis, M., and J.M. Goodman. 1990. Peroxisome assembly: membrane proliferation precedes the induction of the abundant matrix proteins in the methylotrophic yeast Candida boidinii. J. Cell Sci. 96:583–590.

Veenhuis, M., I. Keizer, and W. Harder. 1979. Characterization of peroxisomes in glucose-grown Hansenula polymorpha and their development after the transfer of cells into methanol-containing media. Arch. Microbiol. 120:167–175.

Walton, P.A., P.E. Hill, and S. Subramani. 1995. Import of stably folded proteins into peroxisomes. Mol. Biol. Cell. 6:675–683.

Wilson, L.A., H.L. Niman, R.A. Houghten, A.R. Cheronson, M.L. Connolly, and R.A. Lerner. 1984. The structure of an antigenic determinant in a protein. Cell. 37:767–776.