Yarrowia lipolytica Cells Mutant for the PEX24 Gene Encoding a Peroxisomal Membrane Peroxin Mislocalize Peroxisomal Proteins and Accumulate Membrane Structures Containing Both Peroxisomal Matrix and Membrane Proteins

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Submitted February 28, 2002; Revised April 10, 2002; Accepted May 21, 2002

Monitoring Editor: Reid Gilmore

Peroxisomes are proteins required for peroxisome assembly and are encoded by the PEX genes. Functional complementation of the oleic acid–nonutilizing strain mut1-1 of the yeast Yarrowia lipolytica has identified the novel gene, PEX24. PEX24 encodes Pex24p, a protein of 550 amino acids (61,100 Da). Pex24p is an integral membrane protein of peroxisomes that exhibits high sequence homology to two hypothetical proteins encoded by the open reading frames YHR150W and YDR479C of the Saccharomyces cerevisiae genome. Pex24p is detectable in wild-type cells grown in glucose-containing medium, and its levels are significantly increased by incubation of cells in oleic acid–containing medium, the metabolism of which requires intact peroxisomes. pex24 mutants are compromised in the targeting of both matrix and membrane proteins to peroxisomes. Although pex24 mutants fail to assemble functional peroxisomes, they do harbor membrane structures that contain subsets of peroxisomal proteins.

INTRODUCTION

Peroxisomes belong to the microbody family of organelles, which also includes the glyoxysomes of plants and the glycosomes of trypanosomes. Peroxisomes are spherical in shape, delimited by a single membrane and contain a fine granular matrix and sometimes a paracrystalline core. Peroxisomes inactivate toxic substances, regulate cellular oxygen concentration, and metabolize lipids, nitrogen bases, and carbohydrates (reviewed by Lazarow and Fujiki, 1985; van den Bosch et al., 1992; Subramani, 1998; Purdue and Lazarow, 2001). Peroxisomes are essential for genetic disorders collectively known as the peroxisome biogenesis disorders (PBD), such as Zellweger syndrome, in which peroxisomes fail to assemble properly (Lazarow and Moser, 1994; Subramani, 1998; Subramani et al., 2000; Purdue and Lazarow, 2001).

Defining the molecular bases of the PBDs has been an area of intense research in recent years, particularly in regards to the identification of genes controlling peroxisome assembly. Much progress has been made in the identification of these so-called PEX genes by the use of various yeasts as model systems. To date, the PEX genes for 23 peroxins have been isolated from yeast (for reviews, see Subramani, 1998; Titorenko and Rachubinski, 2001; Purdue and Lazarow, 2001). Thirteen human orthologues of these yeast PEX genes have been identified, and mutations in 11 of these have been shown to cause PBDs (for reviews, see Subramani et al., 2000; Fujiki, 2000; Gould and Valle, 2000).

Protein targeting to peroxisomes is defective in pex mutants. Peroxisomal proteins are encoded by nuclear genes and synthesized on cytosolic polysomes (Lazarow and Fujiki, 1985; Subramani, 1993, 1998; Subramani et al., 2000; Purdue and Lazarow, 2001). Most matrix proteins are targeted to the peroxisome by one of two types of peroxisome targeting signal (PTS). PTS1 is a carboxyl-terminal tripeptide with the consensus sequence (S/A/C)(K/R/H)(L/M) (Gould et al., 1987, 1989, 1990; Aitchison et al., 1991; Swinkels et al., 1992) and is found in the majority of matrix proteins. PTS2 is a sometimes cleaved amino-terminal nonapeptide with the consensus motif (R/K)(L/V/I)X₅(H/Q)(L/A),
which is found in a smaller subset of matrix proteins (Osumi et al., 1991; Swinkels et al., 1991; Glover et al., 1994b; Waterham et al., 1994). A few peroxisomal matrix proteins are targeted by internal PTSs, which remain largely uncharacterized (Purdue and Lazarow, 2001; Titorenko and Rachubinski, 2001). The pathway of targeting proteins to the peroxi-
somal membrane for these receptors (reviewed by Subramani 1998; Hettema et al., 1999; Elgersma et al., 1994). Pex5p and Pex7p are the receptors for PTS1- and PTS2-containing proteins, respectively, and various peroxi-
somes, notably Pex13p and Pex14p, form a docking complex at the peroxisomal membrane for these receptors (reviewed by Subramani 1998; Hettema et al., 1999; Terlecky and Fransen, 2000; Purdue and Lazarow, 2001; Titorenko and Rachubinski, 2001). The pathway of targeting proteins to the peroxi-
somal membrane has been less well defined; however, it appears to be independent of the pathway for matrix protein targeting. Motifs consisting of stretches of basic amino res-

dues have been suggested to target proteins to the peroxi-
somal membrane (McCammon et al., 1994; Dyer et al., 1996; Elgersma et al., 1997; Pause et al., 2000). A distinctive feature of peroxisomes is their ability to import assembled oligo-

ermic proteins (Glover et al., 1994a; McNew and Goodman, 1994; Titorenko et al., 1998, 2002).

Here, we report the isolation and characterization of a novel PEX gene, PEX24, from the yeast Yarrowia lipolytica. Mutants of PEX24 are compromised in peroxisome assembly and mislocalize both peroxisomal matrix and membrane proteins. Pex24p is an integral membrane protein of peroxi-
somes, whose levels are increased by incubation of cells in oleic acid–containing medium.

**MATERIALS AND METHODS**

**Strains and Culture Conditions**

The *Y. lipolytica* strains used in this study are listed in Table 1. All strains were cultured at 30°C. Strains with plasmids were cultured in minimal medium (YNDF or YNDF supplemented with leucine, lysine, and uracil, each at 50 µg/ml, as required).

### Table 1. *Y. lipolytica* strains used in this study

<table>
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<th>Strain</th>
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<td>MATa, ura3-302, leu2-270, lys8-11, pUB4(HygBR)/PEX24</td>
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*Strain E122 was a gift of C. Gaillardin (Thiverval-Grignon, France). All other strains were from this study.*

Cloning, Sequencing, and Integrative Disruption of the PEX24 Gene

The *mut1-1* mutant strain was isolated from randomly mutagenized *Y. lipolytica* wild-type strain E122 as described previously (Nuttley et al., 1993). The PEX24 gene was isolated by functional complementa-
tion of the *mut1-1* strain with a *Y. lipolytica* genomic DNA library in the autonomously replicating *Escherichia coli* shuttle vector pINA445 (Nuttley et al., 1993). *Leu*+ transformants were screened on YNO agar plates for restoration of the ability to use oleic acid as the sole carbon source. Total DNA was isolated from colonies that recovered growth on YNO and used to transform *E. coli* for plasmid recovery. Restriction fragments of the initial complementing genomic insert were subcloned and tested for their ability to com-
plement the *mut1-1* strain. The shortest complementing fragment was sequenced in both directions, and the gene contained therein was designated PEX24.

The *URA3* gene of *Y. lipolytica* was used for targeted integrative disruption of the PEX24 gene. Nine hundred thirty-nine base pairs of DNA immediately upstream of the PEX24 open reading frame (ORF) were amplified by the PCR using the oligonucleotides 5'-ATTGAATTCAGTACCAGTACATGAAAGATC primer (A) and 5'-TGTTAAAGATCGACGAGCATTACATGAAAGATC primer (B), cleaved with EcoRI and Sall, and inserted into the corresponding sites of the vector pGEM4Z (Promega, Madison, WI) to generate the plasmid pUP. Eight hundred forty-six base pairs of DNA immedi-
atly downstream of the PEX24 ORF was amplified by PCR using the oligonucleotides 5’-GACACGTCGACTTTATACAACATT-

GTCGACCC (primer C) and 5’-AAATACGACTTTATACAACATT-

GTCGACCC (primer D), cleaved with Sall and HindIII, and inserted into pUP to produce the plasmid pUP-DS. A 1.7-kbp Sall fragment containing the *Y. lipolytica* *URA3* gene was ligated into the Sall site of pUP-DS. A fragment containing the *URA3* gene flanked by the 939 base pairs of sequence upstream and the 846 base pairs of sequence downstream of the PEX24 ORF was amplified by PCR using primers A and D. This fragment was used to transform *Y. lipolytica* wild-type strain E122 to uracil prototrophy. *Ura* + transfor-
mants were selected and screened for their inability to grow on oleic acid–containing medium. Integration of the *URA3* gene into the correct locus was confirmed by PCR.

**Antibodies**

Antibodies to Pex24p were raised in rabbit and guinea pig against a maltose-binding protein-Pex24p fusion. A *HindIII* fragment enc omplement of the nucleotides 96-1553 of the *PEX24* ORF was cloned into the vector pMAL-c2 (New England Biolabs, Beverly, MA) in-frame and downstream of the ORF encoding maltose-binding protein, followed by expression in *E. coli* (Eitzen et al., 1995). Anti-Pex24p antibodies were affinity purified as described (Crane et al., 1994). Antibodies to the carboxy-terminal SKL tripeptide, isocitrate lyase (ICL), thiolase (THI), acyl-CoA oxidase subunit 5 (AOX), Pex1p, Pex2p, Pex6p, and Kar2p have been de-
scribed previously (Aitchison et al., 1992; Eitzen et al., 1996; Lambkin et al., 2001; Titorenko et al., 1997, 1998, 2000, 2002). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and HRP-conjugated goat anti-guinea pig IgG secondary antibodies (Amersham Biosciences, Baile d’Urfe, Quebec, Canada) were used to detect pri-
mary antibodies in immunoblot analysis. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and rhodamine-conjugated anti-
guinea pig IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used to detect primary antibodies in immunofluo-
rescence microscopy.

**Microscopic Analysis**

Cells were fixed in 3.7% paraformaldehyde for 30 min at room temperature and processed for immunofluorescence microscopy as described (Pringle et al., 1991), except that spheroplasts were pre-
pared by incubation in 0.1 M potassium phosphate, pH 7.5, 1.2 M

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sorbitol, 40 µg of Zymolyase-100T/ml, and 38 mM 2-mercaptoethanol for 15–30 min at 30°C with gentle agitation. Images were captured with a digital fluorescence camera (Spot Diagnostic Instruments, Sterling Heights, MI).

Electron microscopy of whole yeast cells was performed as described previously (Goodman et al., 1990).

**Cell Fractionation and Peroxisome Subfractionation**

Fractionation of oleic acid–induced cells was performed essentially as described (Szilard et al., 1995). Homogenized spheroplasts were subjected to differential centrifugation at 1000 × g for 10 min at 4°C in a JS13.1 rotor (Beckman, Fullerton, CA) to yield a postnuclear supernatant (PNS) fraction. The PNS fraction was subjected to further differential centrifugation at 20,000 × g for 30 min at 4°C to yield a pellet (20KgP) fraction enriched for peroxisomes and mitochondria and a supernatant (20KgS) fraction enriched for cytosol. Peroxisomes were purified from the 20KgP fraction by isopycnic centrifugation on a discontinuous sucrose gradient (Titorenko et al., 1996).

To subfractionate peroxisomes, 10 volumes of ice-cold 0.1 M Na2CO3, pH 11.5, were added to the 20KgP fraction containing 100 µg of protein (Fujiki et al., 1982). The sample was incubated on ice for 45 min with occasional agitation, followed by centrifugation at 200,000 × g for 1 h at 4°C in a TLA120.2 rotor (Beckman) to yield a pellet fraction enriched for integral membrane proteins and a supernatant fraction enriched for soluble proteins.

**Analytical Procedures**

Enzymatic activity of the mitochondrial marker cytochrome c oxidase (Douma et al., 1985) was measured as described. Whole cell lysates were prepared as described (Eitzen et al., 1997). Immunoblotting was performed using a wet transfer system (Ausubel et al., 1994), and antigen-antibody complexes in immunoblots were detected by enhanced chemiluminescence (Amersham Biosciences). Protein concentration was determined using a commercially available kit (Bio-Rad, Mississauga, Ontario, Canada) and bovine serum albumin as a standard. Proteins were precipitated by addition of trichloroacetic acid to 10%, followed by washing of the precipitate with chilled 80% acetone. Oligonucleotides were synthesized on an Oligo 1000 M DNA Synthesizer (Beckman). Sequencing was performed on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA).

**Figure 1.** Growth of various *Y. lipolytica* strains on glucose-containing (YEPD) and oleic acid–containing (YPBO) media. The strains listed in Table 1 were grown to midlog phase in liquid YEPD medium, spotted at dilutions of 10⁻¹ to 10⁻⁵ on both YEPD and YPBO agar, and grown for 5 d at 30°C.

**Figure 2.** Cloning and analysis of the PEX24 gene. (A) Complementing activity of inserts, restriction map analysis, and targeted gene disruption strategy for the PEX24 gene. (B) Nucleotide sequence of the PEX24 gene and deduced amino acid sequence of Pex24p. These sequence data have been deposited in the DDBJ/EMBL/GenBank databases under accession number AF480881.
RESULTS

Isolation and Characterization of the PEX24 Gene

The mut1-1 mutant strain (Table 1) was isolated from randomly mutagenized wild-type Y. lipolytica cells by its inability to grow on medium containing oleic acid as the sole carbon source (the ole phenotype; Figure 1). Morphological and biochemical analyses (data presented below) determined that this strain was defective in peroxisome assembly. The PEX24 gene was isolated from a Y. lipolytica genomic DNA library by functional complementation, i.e., restoration of growth on oleic acid–containing medium (the OLE phenotype) of the mut1-1 strain. DNA was isolated from the complemented strain, and the complementing plasmid was recovered by transformation of E. coli. The complementing fragment, CS-01, was mapped by restriction endonuclease digestion (Figure 2A). Various restriction fragments were subcloned and introduced by transformation into the mut1-1 strain to delineate the region of complementation (Figure 2A). Sequencing of the complementing fragment CS-SS revealed an ORF of 1650 nucleotides encoding a protein of 550 amino acids, Pex24p, with a predicted molecular weight of 61,100 (Figure 2B). Based on algorithms predicting membrane-associated regions in proteins (Eisenberg et al., 1984; Rao and Argos, 1986), Pex24p is predicted to contain two membrane-spanning domains at amino acids 216–249 and 335–361. A search of protein databases with the use of the GENINFO(R) BLAST Network Service of the National Center for Biotechnology Information revealed two proteins encoded by the ORFs YHR150W and YDR479C, respectively, of the S. cerevisiae genome. Amino acid sequences were aligned with the use of the ClustalW program (EMBL, Heidelberg, Germany). Identical residues (black) and similar residues (gray) in at least two of the proteins are shaded. Similarity rules: G = A; A = V; V = L = M; I = L = M = F = Y = W; K = R = H; D = E = Q = N; and S = T = Q = N. Dashes represent gaps.

Figure 3. Sequence alignment of Pex24p with the hypothetical proteins Yhr150wp and Ydr479cp encoded by the ORFs YHR150W and YDR479C, respectively, of the S. cerevisiae genome. Amino acid sequences were aligned with the use of the ClustalW program (EMBL, Heidelberg, Germany). Identical residues (black) and similar residues (gray) in at least two of the proteins are shaded. Similarity rules: G = A; A = V; V = L = M; I = L = M = F = Y = W; K = R = H; D = E = Q = N; and S = T = Q = N. Dashes represent gaps.
gene in the mut1-1 strain revealed a nonsense mutation at codon 118.

The PEX24 gene was deleted by targeted integration of the Y. lipolytica URA3 gene to generate the strain pex24KOA (Table 1). This strain was unable to grow on oleic acid–containing medium (Figure 1) and showed similar morphological and biochemical defects to the original mut1-1 strain (see below).

pex24 Cells Lack Normal Peroxisomes and Mislocalize Peroxisomal Proteins to the Cytosol

In electron micrographs, normal peroxisomes of Y. lipolytica grown in oleic acid–containing medium appear as round vesicular structures, 0.2–0.5 μm in diameter, surrounded by a single unit membrane and containing an homogenous granular matrix (Figure 4A). The original mutant strain mut1-1 (Figure 4B) contained small vesicular structures and some larger vesicles resembling peroxisomes and accumulated membranous sheets around the nucleus that were rarely seen in wild-type cells. The deletion strain pex24KOA showed no morphologically recognizable peroxisomes but again showed an accumulation of extended membranes (Figure 4C). Strain P24TR transformed with the PEX24 gene had the appearance of the wild-type strain and showed normal peroxisome morphology (Figure 4D).

Figure 4. Ultrastructure of wild-type, pex24 mutant, and PEX24-transformed strains. The E122 (A), mut1-1 (B), pex24KOA (C), and P24TR (D) strains were grown in glucose-containing YEPD medium for 16 h, shifted to oleic acid–containing YPBO medium and incubated for an additional 9 h. Cells were fixed in 1.5% KMnO4 and processed for electron microscopy. L, lipid droplet; M, mitochondrion; N, nucleus; P, peroxisome. Bar, 1 μm.
oxisomal staining pattern observed in wild-type cells, indicating the ability of this gene to rescue the import of these peroxisomal proteins.

Cells of the wild-type strain E122 and of the mutant strains mut1-1 and pex24KO were grown for 16 h in glucose-containing medium, shifted to oleic acid–containing medium for an additional 9 h, and then fractionated into a 20,000 g pellet (20KgP) fraction enriched for peroxisomes and mitochondria and a 20,000 g supernatant (20KgS) fraction enriched for cytosol. In agreement with data from immunofluorescence microscopy, peroxisomal matrix proteins (Figure 6) were preferentially localized to the 20KgP fraction of wild-type cells; however, they were localized primarily to the 20KgS fraction of both mutant strains. It is noteworthy that AOX was found equally distributed between the 20KgS and 20KgP fractions of the original mutant strain mut1-1. The mitochondrial marker enzyme cytochrome c oxidase was preferentially localized to the 20KgP fraction of all strains. Because in pex24 mutant strains all matrix proteins investigated mislocalized preferentially to the 20KgS fraction enriched for cytosol and exhibited a generalized pattern of fluorescence characteristic of the cytosol in immunofluorescence microscopy, pex24 mutant cells are compromised in the import of PTS1 (ICL and anti-SKL proteins), PTS2 (THI), and non-PTS1, non-PTS2 proteins (AOX; Wang et al., 1999). The peroxisomal peroxin Pex19p (Lambkin and Rachubinski, 2001), the peripheral peroxisomal membrane peroxin Pex16p (Eitzen et al., 1997) and the integral peroxisomal membrane protein Pex2p. Rabbit primary antibodies (SKL, AOX, and ICL) were detected with fluorescein-conjugated secondary antibodies. Guinea pig primary antibodies (THI and Pex2p) were detected with rhodamine-conjugated secondary antibodies. Bar, 1 μm.

pex24 Cells Contain Membrane Structures Containing Both Peroxisomal Matrix and Membrane Proteins

The 20KgP fractions of the wild-type strain E122 and of the mut1-1 and pex24KO mutant strains incubated in oleic acid–containing medium were subjected to isopycnic sucrose gradient density centrifugation. Fractions were analyzed by immunoblotting with antibodies to peroxisomal matrix proteins (anti-SKL proteins, ICL, AOX, and THI), to the peroxins Pex16p, Pex19p, and Pex2p, and to the endoplasmic reticulum-resident protein, Kar2p (Figure 7). In E122 cells, all peroxisomal proteins investigated were found primarily in fractions 3–5, peaking in fraction 4 at a density of 1.21 g/cm3, which has previously been reported as the density of peroxisomes of Y. lipolytica in sucrose (Szilard et al., 1995; Titorenko et al., 1996; Brown et al., 2000), whereas Kar2p

Figure 5. Peroxisomal matrix and membrane proteins are mislocalized in pex24 mutant strains. Wild-type strain E122, mutant strains mut1-1 and pex24KO, and transformed strain P24TR were grown in YEPD medium for 16 h, transferred to YPBO medium, and incubated for an additional 9 h. Cells were processed for immunofluorescence microscopy with antibodies to the PTS1 tripeptide SKL (SKL), 1’-acyl-CoA oxidase (AOX), isocitrate lyase (ICL), thiolase (THI), and the integral peroxisomal membrane protein Pex2p. Rabbit primary antibodies (SKL, AOX, and ICL) were detected with fluorescein-conjugated secondary antibodies. Guinea pig primary antibodies (THI and Pex2p) were detected with rhodamine-conjugated secondary antibodies. Bar, 1 μm.
Peroxisomal matrix proteins and peroxisomal peroxins are found in the wild-type strain. In the mutant strain, Pex24p was localized exclusively to the 20KgP fraction enriched for peroxisomes and mitochondria from wild-type cells (Figure 8B) and fractionated with peroxisomes in isopycnic density gradient centrifugation (Figure 7). Lysis of peroxisomes with alkali Na2CO3 followed by high-speed centrifugation, showed that Pex24p cofractionated with Pex2p to the pellet fraction enriched for integral membrane proteins (Figure 8C). Therefore, Pex24p is an integral membrane protein of peroxisomes.

**Synthesis of Pex24p Is Induced by Incubation of Cells In Oleic Acid-Containing Medium**

Wild-type E122 cells grown in glucose-containing medium were transferred to oleic acid–containing YPBO medium and incubated for 8 h in this medium. Aliquots of cells were removed at various times during the incubation in YPBO medium, and their lysates were subjected to SDS-PAGE and immunoblotting. Pex24p was barely detectable at the time of transfer to YPBO medium, but its synthesis increased with time after the transfer (Figure 9). Under the same conditions, the level of the peroxisomal matrix enzyme thiolase (THI) increased dramatically, whereas the level of the cytosolic enzyme glucose-6-phosphate dehydrogenase (G6PDH) remained unchanged.

**DISCUSSION**

We have identified and characterized a novel mutant of peroxisome assembly of the yeast *Y. lipolytica*. Functional complementation of this mutant strain has led to the identification of the gene *PEX24*, which encodes a 550-amino acid protein, Pex24p, with a predicted molecular mass of 61,100 Da. Pex24p was shown to be peroxisomal by both double-label, indirect immunofluorescence microscopy and subcellular fractionation. Pex24p is predicted to contain two membrane-spanning domains and displays the characteristics of an integral membrane protein during extraction of a subcellular fraction enriched for peroxisomes with alkali sodium carbonate. Pex24p shows strong sequence similarity to two putative proteins encoded by the ORFs YHR150W and YDR479C of the *S. cerevisiae* genome. These proteins, which have not yet been characterized, are the subject of current investigations in our laboratory. Possible functional redundancy between these two proteins may have prevented their ready identification as *PEX* genes in *S. cerevisiae* by selection procedures involving random mutagenesis.

The ability to use oleic acid as sole carbon source was greatly reduced in the original mutant strain *mut1-1*, whereas it was completely abolished in the deletion strain *pex24KO*. DNA sequencing revealed a nonsense mutation at codon 118 of the *PEX24* gene of the *mut1-1* strain. Judging from the reduced growth of the *mut1-1* strain on oleic acid–containing medium and the presence of small vesicular structures resembling peroxisomes in *mut1-1* cells seen by electron microscopy, it can be speculated that the shortened form of Pex24p synthesized in the *mut1-1* strain retains some of its function(s).

Isopycnic density gradient centrifugation analysis showed that both the original mutant strain *mut1-1* and the deletion strain *pex24KO* contain membrane structures having densities both like and less than that of normal peroxisomes. These membrane structures are not "peroxisome ghosts," which are found in cells of Zellweger syndrome patients and

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**Figure 6.** Peroxisomal matrix proteins and peroxisomal peroxins show mislocalization in *pex24* mutant strains. The wild-type strain E122, the original mutant strain *mut1-1*, and the deletion strain *pex24KO* were grown in glucose-containing YEPD medium for 16 h, transferred to oleic acid–containing YPBO medium, and incubated for an additional 9 h. Cells were subjected to subcellular fractionation to yield a 20KgP fraction enriched for peroxisomes and mitochondria and a 20KgS fraction enriched for cytosol. Equal portions of the 20KgS and 20KgP were analyzed by immunoblotting to peroxisomal matrix proteins (SKL, ICL, AOX, and THI) and to peroxisomal peroxins.

Exhibited an almost even distribution across all gradient fractions. In *mut1-1* and *pex24KO* cells, evidence of membrane structures having a density similar to that of wild-type peroxisomes was observed; however, these structures contained a complement of proteins different from that of wild-type peroxisomes. Anti-SKL proteins, ICL, THI, and Pex19p, but not the peripheral membrane protein Pex16p or the integral membrane protein Pex2p, were detected in structures found in fraction 4 of the *mut1-1* strain. Only anti-SKL proteins, ICL and THI, were detected in structures found in fraction 4 of the deletion strain *pex24KO*. Membrane structures of density less than that of wild-type peroxisomes but containing peroxisomal proteins were also observed for both wild-type cells and to a much greater extent for *mut1-1* and *pex24KO* cells. The origin of these membrane structures is currently unknown, but it should be noted that Kar2p is involved in the assembly of peroxisome ghosts, whereas only the precursor form was detected in the *pex24KO* deletion strain. For all strains, the mitochondrial marker cytochrome c oxidase localized primarily to fractions 9 and 10 with densities both like and less than that of normal peroxisomes. These membrane structures are not "peroxisome ghosts," which are found in cells of Zellweger syndrome patients and...
were defined originally as membranous structures containing peroxisomal membrane proteins but not peroxisomal matrix proteins (Santos et al., 1988), because they contain both peroxisomal matrix and membrane proteins. Similar membrane structures have been reported for other Y. lipolytica pex strains (Brown et al., 2000; Lambkin and Rachubinski, 2001). Whether these structures are precursors to mature peroxisomes (South and Gould, 1999; Titorenko et al., 2000) or simply types of peroxisomes lacking their full complement of peroxisomal proteins is unknown at present.

Pex5p and Pex7p act as cytosolic receptors for PTS1- and PTS2-containing proteins, respectively. Although there is distinct separation in these two pathways of matrix protein import at this initial stage, convergence of the two pathways is believed to occur at the level of the peroxisome and to involve the peroxins Pex13p and Pex14p. Pex13p and Pex14p are integral proteins of the peroxisomal membrane that recognize both Pex5p and Pex7p and form a complex with each other (for reviews, see Subramani, 1998; Hettema et al., 1999; Purdue and Lazarow, 2001; Titorenko and Rachubinski, 2001). Because the import of all peroxisomal matrix proteins investigated in this study is compromised in the pex24 mutant strains regardless of their type of PTS, Pex24p may act downstream of the point of convergence of the PTS1 and PTS2 pathways, thereby affecting the import of all peroxisomal matrix proteins. It should be noted that the targeting of peroxisomal membrane proteins is also compromised in pex24 mutant strains. Because the targeting of peroxisomal matrix and membrane proteins apparently occurs by independent pathways and mechanisms (for reviews, see Subramani, 1998; Hettema et al., 1999; Purdue and Lazarow, 2001; Titorenko and Rachubinski, 2001), the primary role of Pex24p may actually be in the targeting and/or assembly of peroxisomal membrane proteins. The effects of mutation of Pex24p on peroxisomal matrix protein import would therefore be secondary to the primary defect in peroxisomal

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Figure 7. Peroxisomal proteins of pex24 cells localize in part to membrane structures that are of the same density as wild-type peroxisomes. The 20KgP fractions of the wild-type strain E122, the original mutant strain mut1-1 and the deletion strain pex24KOA incubated in oleic acid–containing medium for 9 h were subjected to isopycnic centrifugation on discontinuous sucrose gradients. Fifteen 2-ml fractions were collected from the bottom of each tube. Equal volumes of each fraction were analyzed by SDS-PAGE and subjected to immunoblotting with antibodies to the indicated proteins. The volume of fractions of the mut1-1 and pex24KOA strains analyzed by SDS-PAGE was 10 times that of fractions of the wild-type strain E122.
membrane protein targeting/assembly. Dysfunction and/or absence of Pex24p could also be proposed to lead to major structural alterations in the peroxisomal membrane that would prevent or hinder the correct assembly of the translocation machineries required for the import of matrix and membrane proteins. Defining the exact role played by Pex24p in the peroxisome assembly process awaits further experimentation, including an analysis of the interacting partners of Pex24p.

In conclusion, we have identified and characterized a novel peroxin, Pex24p, required for peroxisome assembly in the yeast Y. lipolytica. Pex24p is an integral peroxisomal membrane protein. Mutants of the PEX24 gene are compromised in the targeting of both peroxisomal matrix and membrane proteins and fail to assemble functional peroxisomes, but they are nevertheless capable of assembling membrane structures that exhibit peroxisomal characteristics.

ACKNOWLEDGMENTS

We thank Honey Chan for help with electron microscopy and Richard Poirier for expert technical assistance. This work was supported by grant MOP-9208 from the Canadian Institutes of Health Research (CIHR) to R.A.R. R.A.R. is Canada Research Chair in Cell Biology, a Senior Investigator of the CIHR, and an International Research Scholar of the Howard Hughes Medical Institute.

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