***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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Peroxisins 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),

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Abbreviations used: 20KgP, 20,000 × g pellet; 20KgS, 20,000 × g supernatant; AOX, acyl-CoA oxidase; ICL, isocitrate lyase; ORF, open reading frame; PBD, peroxisome biogenesis disorder; *pex*, peroxisome assembly mutant; *PEX*, gene encoding a peroxin; PNS, postnuclear supernatant; PTS, peroxisome targeting signal; THI, thiolase.

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

Strain ^a	Genotype
E122	MATA, <i>ura3-302</i> , <i>leu2-270</i> , <i>lys8-11</i>
<i>mut1-1</i>	MATA, <i>ura3-302</i> , <i>leu2-270</i> , <i>lys8-11</i> , <i>ole</i>
<i>pex24KOA</i>	MATA, <i>ura3-302</i> , <i>leu2-270</i> , <i>lys8-11</i> , <i>pex24::URA3</i>
P24TR	MATA, <i>ura3-302</i> , <i>leu2-270</i> , <i>lys8-11</i> , <i>pUB4(HygBR)PEX24</i>

^a Strain E122 was a gift of C. Gaillardin (Thiverval-Grignon, France). All other strains were from this study.

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 *et al.*, 1990; Kragler *et al.*, 1993; Elgersma *et al.*, 1995). Pex5p and Pex7p are the receptors for PTS1- and PTS2-containing proteins, respectively, and various peroxins, 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 peroxisomal 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 residues have been suggested to target proteins to the peroxisomal 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 oligomeric 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 peroxisomes, 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 (YND or YNO), except for strain P24TR, which expresses the PEX24 gene from the plasmid pUB4 (Kerscher *et al.*, 2001) and was cultured in YEPD or YPBO supplemented with Hygromycin B (Sigma, St. Louis, MO) at a concentration of 125 µg/ml. Media components were as follows: YEPD, 1% yeast extract, 2% peptone, 2% glucose; YPBO, 0.3% yeast extract, 0.5% peptone, 0.5% K₂HPO₄, 0.5% KH₂PO₄, 1% Brij 35, 1% (vol/vol) oleic acid; YND, 0.67% yeast nitrogen base without amino acids, 2% glucose; YNO, 0.67% yeast nitrogen base without amino acids, 0.05% (wt/vol) Tween 40, 0.1% (vol/vol) oleic acid. YND and YNO were supplemented with leucine, lysine, and uracil, each at 50 µg/ml, as required.

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 complementation 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 complement 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'-TGATATAAAGTCGACGTGTGCGGGTGGTTGTGT (primer B), cleaved with *Eco*RI and *Sal*I, and inserted into the corresponding sites of the vector pGEM4Zf (Promega, Madison, WI) to generate the plasmid pUP. Eight hundred forty-six base pairs of DNA immediately downstream of the PEX24 ORF was amplified by PCR using the oligonucleotides 5'-GCACACGTCGACTTTATACAACATTGTCGAGCG (primer C) and 5'-ATTAAGCTTGTGCGGTGTCGAGAC (primer D), cleaved with *Sal*I and *Hind*III, and inserted into pUP to produce the plasmid pUP-DS. A 1.7-kbp *Sal*I fragment containing the *Y. lipolytica* URA3 gene was ligated into the *Sal*I 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⁺ transformants 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 *Hind*III fragment encompassing nucleotides 969-1653 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 carboxyl-terminal SKL tripeptide, isocitrate lyase (ICL), thiolase (THI), acyl-CoA oxidase subunit 5 (AOX), Pex1p, Pex2p, Pex6p, Pex16p, Pex19p, Pex20p, and Kar2p have been described 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, Baie d'Urfé, Quebec, Canada) were used to detect primary 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 immunofluorescence 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 prepared by incubation in 0.1 M potassium phosphate, pH 7.5, 1.2 M

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.

sorbitol, 40 μ g 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 $1000 \times g$ 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 $20,000 \times g$ 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 Na_2CO_3 , pH 11.5, were added to the 20K_{GP} 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 $200,000 \times g$ 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). Extraction of nucleic acid from yeast lysates and manipulation of DNA were performed as described (Ausubel *et al.*, 1994). 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 (BioRad, 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 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. The thick black line represents the original complementing insert DNA. (Solid lines) *Y. lipolytica* genomic DNA; (dotted lines) vector DNA. The ORFs of the *PEX24* and *URA3* genes and their directionality are denoted by the wide arrows. (+) Ability and (–) inability of an insert to confer growth on oleic acid to strain *mut1-1*. (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.

Pex24p	1	M--FRRLTGSQSSSSHYGLVNDSTQTHGTYDANALASCLDMLKKDKPLSDDDSAEVAE
Yhr150wp	1	MSETSSRRRSASKDAKGYFACKYKVLDSIIEAARISKPTVAEDLGGSSSSGNSEMS
Ydr479cp	1	M-----DSVTFNFTNDTNACTPFRSTLKGGKVONIDEKSKAKKESISSGRTSDPT
Pex24p	59	SEASRTSLGSPITP--HSHFVDALYDKVILPKLKKSIIGDVSDLGSLVATPTGCSR
Yhr150wp	61	EPSTLASSATSGQISKELLQCIACSLFSTSTERLKTSHSEVSTPEYVNDSYGEQEC
Ydr479cp	54	RGLSPSSSGGPTSGGGFPSTENIQRMADTLVERKIRHALPPSEKTAVDITTHRVAGKE
Pex24p	118	RGSFSTIRALDISE--EPFSESISVFDSDSVASTASSEPFPRISQHPKPKVDFPEAHE
Yhr150wp	121	RECDGVKRCSAHFEGAPEHYLDSTESGPALFPHTENSEKDPFIDVFLKLSRIVPEKLP
Ydr479cp	114	R-----
Pex24p	176	AG-----PELSPLLENNIAELSKRAGGVHKTQITVWLRAIQWQNPCL
Yhr150wp	181	EREHFSKTTIEHDLDTGRVDFESATLTCNFKKLSKKMGSIIFLDQSTIVRLTLTRNPTG
Ydr479cp	115	-----PKLSVQITSENFNTQMNLSRLGVPFMMDELKILNNTNPAN
Pex24p	218	SISCHFYTTANCLYP-GIVFVLPGLLFHYGIMAPYAEKHPLPEYRPEPPTVKN----
Yhr150wp	241	TVTSILLETLLICNMP-MYLVILPIRFVYGIWVPGYVRKHPLQSSHYELKRNHGSLLYD
Ydr479cp	155	TVSILFLYTLILRPFCQLSSLPFIFLLLCVMVPCYLYIHKP-N---PISLIDNN----
Pex24p	272	-----EFDMAADDTSIRAAGIKRKAARE-----KPVDDKKSKMNF
Yhr150wp	300	VCYEGKNEYSYGQQFESKFFPDLESRNCEIDEISELDRRTENTGELKQGHKVLINLRDM
Ydr479cp	206	-----QHPAGPILRRPEVPRVPELS-----QEVFLNLTDL
Pex24p	309	QNATINLIKALDKIESFLSGPAGFANESLSALIFLLIGRAMIGTFLS----AFIPWKA
Yhr150wp	360	QNTTSGILHVIEANSEFLRSESSFCNEECSTKRFTGFLIVFLKILS----PFVNUSYV
Ydr479cp	238	QNHMLLVKVFHFTLLILQRFAPFTNEAISSEFYFIVLLIATLNFLYMDKFIKILIPRPV
Pex24p	365	CTGAGMGVITAGHPTLLKRIVAILLELEKSDNEEKPKESPVIATLCKVCKEFINDEPPC
Yhr150wp	416	CSIFAUCLLYHHPRAPKPIISFFKGTGCKEYKULK-----REHCALNMIFDEQPE
Ydr479cp	298	LILLGMGFFIASHPENFEYLLLRNSEEIRLKTITSTI-----LESILQHKLTARE
Pex24p	425	GETVEIFELQRCGLTPRCQDSVFTSLVYDLCSURCAKKRPP--GAPFLGDVEAPEGWL
Yhr150wp	469	TQTEIFEIYKRAILLNDVRFERYSNRIEDPDDYRRACQEPF--GVDSLADVIPPITGWS
Ydr479cp	353	HELVMIFEIQYLPVEYKEUPVCFSDDDSLFSSLRIVCRRIEENSVKSLTEEPPKDMF
Pex24p	483	FSKMHDIEVDNNNEVWGERGLGRCTEDNDEEVYDV--EKGVGEVRRRRFIRTAKEST
Yhr150wp	527	FDPNFEKIKDNDVDRWVERGLN----LPITG-----EFLFDPMFRRRLIIRVINKAT
Ydr479cp	413	DEANSHVLDLDRPWEVEFLICVYEIDSETRWYVDNLDCQRCSTYRRRMITNSCVKKL
Pex24p	542	VPEKLLGLV-----
Yhr150wp	577	PVL-----
Ydr479cp	473	DSGISNIGEEEVVNPLREETYRQGVHGVTKGSMGGGLTHSSDDDRADEESINGTIPNLN
Pex24p		-----
Yhr150wp		-----
Ydr479cp	533	NIDADASYPSSIEELDTLNSTI

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 = S; A = V; V = I = L = M; I = L = M = F = Y = W; K = R = H; D = E = Q = N; and S = T = Q = N. Dashes represent gaps.

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* of the *Saccharomyces cerevisiae* genome (Figure 3) that share extensive sequence homology with Pex24p. Sequencing of the *PEX24*

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% KMnO_4 and processed for electron microscopy. L, lipid droplet; M, mitochondrion; N, nucleus; P, peroxisome. Bar, 1 μm .

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).

Immunofluorescence analysis of oleic acid-incubated wild-type *E122* cells with anti-SKL antibodies and antibodies to the matrix proteins acyl-CoA oxidase (AOX), isocitrate lyase (ICL), and thiolase (THI) and to the peroxisomal integral membrane protein Pex2p showed a punctate pattern of staining characteristic of peroxisomes (Figure 5). In contrast, *mut1-1* and *pex24KOA* cells stained with the same antibodies showed a more diffuse pattern of fluorescence characteristic of a cytosolic localization (Figure 5). Strain *P24TR* transformed with the *PEX24* gene showed the characteristic per-

Figure 5. Peroxisomal matrix and membrane proteins are mislocalized in *pex24* mutant strains. Wild-type strain *E122*, mutant strains *mut1-1* and *pex24KOA*, 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), 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 μ m.

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 *pex24KOA* 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 $20,000 \times g$ pellet (20KgP) fraction enriched for peroxisomes and mitochondria and a $20,000 \times g$ 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* mutants 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 (Lam-

bin and Rachubinski, 2001), the peripheral peroxisomal membrane peroxin Pex16p (Eitzen *et al.*, 1997) and the integral peroxisomal membrane peroxin Pex2p (Eitzen *et al.*, 1996) were all localized primarily to the 20KgP of *E122* cells (Figure 6). In contrast, these peroxins were localized almost exclusively to the 20KgS of both *mut1-1* and *pex24KOA* cells, demonstrating a preferential mislocalization of these peroxisomal peroxins to the cytosol in these strains.

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/cm^3 , 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 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 *pex24KOA* 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 *pex24KOA* 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 *pex24KOA*. 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 *pex24KOA* cells. The origin of these membrane structures is currently unknown, but it should be noted that Kar2p is readily seen to cofractionate with them in gradients of the *mut1-1* and *pex24KOA* strains. It is noteworthy that both the 47-kDa precursor form and the 45-kDa mature form of thiolase were detected in the *mut1-1* strain, whereas only the precursor form was detected in the *pex24KOA* deletion strain. For all strains, the mitochondrial marker cytochrome *c* oxidase localized primarily to fractions 9 and 10 with densities of 1.18 and 1.17 g/cm³, well separated from fraction 4 containing the peak immunodetection of peroxisomal proteins for the wild-type strain.

Pex24p Is an Integral Membrane Protein of Peroxisomes

Double-labeling indirect immunofluorescence microscopy of wild-type cells incubated in oleic acid-containing medium with antibodies to thiolase and to Pex24p showed colocalization of these proteins to punctate structures (Figure 8A). 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 proteins 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 *pex24KOA*. 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 *pex24KOA* 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

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.

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

Figure 8. Pex24p is an integral peroxisomal membrane protein. (A) Double-labeling, indirect immunofluorescence microscopy of wild-type cells with antibodies to thiolase (THI) and to Pex24p. Bar, 1 μ m. (B) Immunoblot analysis of 20KgS and 20KgP subcellular fractions from wild-type cells incubated in oleic acid-containing medium with anti-Pex24p antibodies. Equivalent portions of each fraction were analyzed. (C) Immunoblot analysis of wild-type peroxisomes treated with alkali Na_2CO_3 and separated by centrifugation into supernatant (S) and pellet (P) fractions. The top and second blots were probed with antibodies to THI and acyl-CoA oxidase (AOX), respectively, to detect peroxisomal matrix proteins. The third blot was probed with antibodies to the peroxisomal integral membrane protein Pex2p. The bottom blot was probed with antibodies to Pex24p. Equivalent portions of the supernatant and pellet fractions were analyzed.

Figure 9. Synthesis of Pex24p is induced by incubation of *Y. lipolytica* in oleic acid-containing medium. Wild-type E122 cells grown for 16 h in glucose-containing YEPD medium were transferred to, and incubated in, oleic acid-containing YPBO medium. Aliquots of cells were removed from the YPBO medium at the times indicated, and total cell lysates were prepared. Equal amounts of protein from the total cell lysates were analyzed by SDS-PAGE and immunoblotting with antibodies to Pex24p, thiolase (THI), and glucose-6-phosphate dehydrogenase (G6PDH).

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.

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