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The *Yarrowia lipolytica* Gene *PAY5* Encodes a Peroxisomal Integral Membrane Protein Homologous to the Mammalian Peroxisome Assembly Factor PAF-1*

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***Pay* mutants of the yeast *Yarrowia lipolytica* fail to assemble functional peroxisomes. One mutant strain, *pay5-1*, lacks normal peroxisomes and instead contains irregular vesicular structures surrounded by multiple unit membranes. The *pay5-1* mutant is not totally deficient in peroxisomal matrix protein targeting, as a subset of matrix proteins continues to localize to a subcellular fraction enriched for peroxisomes. The functionally complementing gene *PAY5* encodes a protein, Pay5p, of 380 amino acids (41,720 Da). Pay5p is a peroxisomal integral membrane protein homologous to mammalian PAF-1 proteins, which are essential for peroxisome assembly and whose mutation in humans results in Zellweger syndrome. Pay5p is targeted to mammalian peroxisomes, demonstrating the evolutionary conservation of the targeting mechanism for peroxisomal membrane proteins. Our results suggest that in *pay5* mutants, normal peroxisome assembly is blocked, which leads to the accumulation of the membranous vesicular structures observed.**

Eukaryotic cells have evolved a complex set of organelles, with each organelle possessing a specific complement of enzymes required for its particular metabolic role. This compartmentalization of biochemical functions permits a level of metabolic control unavailable to prokaryotes. However, it presents the eukaryotic cell with the problem of directing proteins from their sites of synthesis in the cytosol to their specific organellar locations. Accordingly, eukaryotic cells have developed mechanisms for recognizing newly made organellar proteins and for targeting them to their correct destinations.

In the case of peroxisomes, two types of peroxisomal targeting signals (PTS)[1] act to direct proteins to the peroxisome matrix. Many peroxisomal matrix proteins are targeted by carboxyl-terminal tripeptide motifs called PTS1, which are identical to, or conserved variants of, the prototypical Ser-Lys-Leu PTS1 found in firefly luciferase. A second type of targeting signal, PTS2, is found at the amino termini of mammalian and yeast thiolases and at the amino termini of a limited number of other peroxisomal (microbody) proteins. A small number of peroxisomal matrix proteins are targeted by largely uncharacterized internal PTSs. Peroxisomal membrane proteins have neither PTS1 nor PTS2 sequences but contain PTSs that have been defined only as rather large internal portions of the proteins (for reviews on the various PTSs, see Refs. 1–4, and references cited therein).

Yeast represent an excellent experimental system by which genes encoding the proteins required for peroxisomal protein targeting and peroxisome assembly can be identified. Peroxisome assembly mutants have been isolated in *Saccharomyces cerevisiae* (5–7), *Hansenula polymorpha* (8), *Pichia pastoris* (9, 10), and *Yarrowia lipolytica* (11). Functional complementation of some of these mutants have identified genes encoding a varied set of proteins required for peroxisome biogenesis (for a recent review, see Ref.4), including proteins required for the recognition, targeting, and translocation of PTS1-containing (12–20) and PTS2-containing (21–23) peroxisomal matrix proteins.

Here we report the morphological and initial biochemical characterization of a peroxisome assembly mutant of *Y. lipolytica*, *pay5-1*. *Pay5-1* fails to assemble normal peroxisomes but does accumulate vesicular structures surrounded by multiple membranes. Functional complementation of the *pay5-1* mutant yielded the *PAY5* gene, which encodes Pay5p, a peroxisomal integral membrane protein that contains a C_3HC_4-RING-finger domain (24). Pay5p is homologous to mammalian PAF-1 proteins, which are required for peroxisome assembly and whose mutation results in the hereditary human peroxisome assembly disorder, Zellweger syndrome (25, 26).

MATERIALS AND METHODS

Yeast Strains and Microbial Techniques—The *Y. lipolytica* strains used in this study are listed in Table I. Media, growth conditions, and genetic techniques have been described (11, 20). Standard recombinant DNA techniques were performed as described previously (27).

Cloning, Sequencing and Integrative Disruption of the PAY5 Gene—The mutant *pay5-1* was isolated as described elsewhere (11). The *PAY5* gene was cloned from a *Y. lipolytica* genomic library in the vector pINA445 (11) by functional complementation of the *pay5-1* mutation. Leu^+ transformants were screened for restoration of growth on oleic acid as the carbon source (ole^+ phenotype).

Overlapping restriction endonuclease fragments of the *PAY5* gene were cloned into the vectors pGEM5Zf(+) and pGEM7Zf(+) (Promega, Madison, WI) for dideoxynucleotide sequencing of both strands.

Integrative disruption of the *PAY5* gene was accomplished by replac-

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[1] The abbreviations used are: PTS, peroxisomal targeting signal; CHO, Chinese hamster ovary; 20KgP, 20,000 × *g* pellet (enriched for peroxisomes and mitochondria); 20KgS, 20,000 × *g* supernatant (enriched for cytosol); bp, base pair(s); kbp, kilobase pair(s).

TABLE I
Y. lipolytica strains used in this study

Strain	Genotype
E122	*MATA,ura3–302,leu2–270,lys8–11*
22301–3	*MATB,ura3–302,leu2–270,his1*
pay5–1	*MATA,ura3–302,leu2–270,lys8–11,pay5–1*
PAY5	*MATA,ura3–302,leu2–270,lys8–11,pay5–1*,pBH7*(LEU2)*
P5KO-8A	*MATA,ura3–302,leu2–270,lys8–11,pay5::LEU2*
P5KO-9B	*MATB,ura3–302,leu2–270,his1,pay5::LEU2*
D5-OB	*MATA/MATB,ura3–302/ura3–302,leu2–270/leu2–270,lys8–11/+,+/his1,pay5–1/+*
D5-O9	*MATA/MATB,ura3–302/ura3–302,leu2–270/leu2–270,lys8–11/+,+/his1,pay5–1/pay5::LEU2*
D5-A9	*MATA/MATB,ura3–302/ura3–302,leu2–270/leu2–270,lys8–11/+,+/his1,+/pay5::LEU2*
D5–8B	*MATA/MATB,ura3–302/ura3–302,leu2–270/leu2–270,lys8–11/+,+/his1,pay5::LEU2/+*
D5–89	*MATA/MATB,ura3–302/ura3–302,leu2–270/leu2–270,lys8–11/+,+/his1,pay5::LEU2/pay5::LEU2*
D1-AB	*MATA/MATB,ura3–302/ura3–302,leu2–270/leu2–270,lys8–11/+,+/his1*

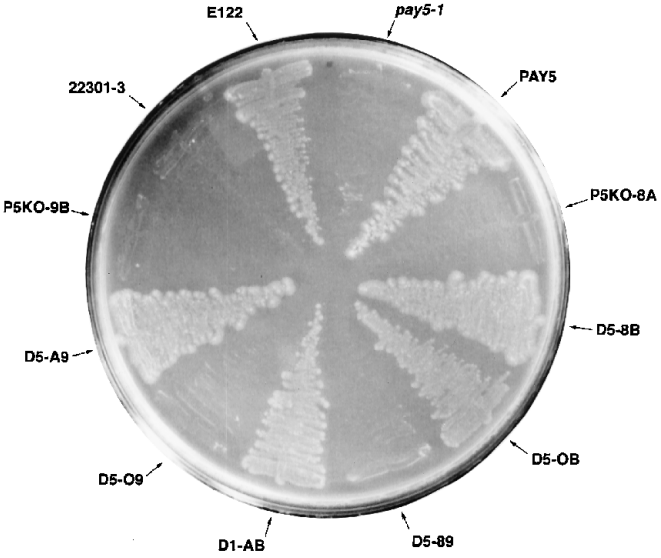

FIG. 1. **Growth of various *Y. lipolytica* strains on oleic acid medium.** The strains listed in Table I were grown for 4 days on YNO-agar (YNO = 0.67% yeast nitrogen base without amino acids, 0.05% (v/v) Tween 40, 0.1% (w/v) oleic acid). The appearance of the complemented strain *PAY5* is compared to that of the wild-type strain *E122*, the original *pay5-1* mutant, the gene disruption strains *P5KO-8A* and *P5KO-9B*, and the diploid strains *D5-A9*, *D5-O9*, *D1-AB*, *D5–89*, *D5-OB*, and *D5-8B*. The parental strain *22301-3* was not supplemented for its auxotrophic requirements. Growth on YNO requires at least one intact copy of the *PAY5* gene.

ing a 1480-bp *Sna*BI/*Bgl*II fragment, which included most of the open reading frame of the *PAY5* gene, with a 2.1-kbp *Eco*47III/*Bgl*II fragment from pINA445 containing the *LEU2* gene of *Y. lipolytica*. The construct was digested with *Bam*HI and *Hin*dIII to liberate the *LEU2* gene flanked by 125 and 423 bp of the *PAY5* gene at its 5′ and 3′ ends, respectively. This linear DNA fragment was used to transform *Y. lipolytica* wild-type strains *E122* and *22301-3* to leucine prototrophy to make the strains *P5KO-8A* and *P5KO-9B*, respectively. Leu^+ transformants were screened for the ole^- phenotype. Integration of the *LEU2* gene at the *PAY5* locus was confirmed by Southern blotting.

Organelle Isolation and Subfractionation—Fractionation of oleic acid-grown *Y. lipolytica* cells into an organellar pellet (20KgP) enriched for peroxisomes and mitochondria and a supernatant (20KgS) enriched for cytosol, and peroxisome purification from the 20KgP were performed as described previously (20). Peroxisome subfractionation and extraction were performed as described previously (20). Essentially, peroxisomes from the fraction of peak peroxisomal enzymatic activity were lysed by the addition of 10 volumes of ice-cold Ti8 buffer (10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin/ml, 1 μg of aprotinin/ml) (28), followed by incubation on ice for 15 min with occasional agitation. The suspension was centrifuged at 200,000 × g_{av} for 1 h at 4 °C in a Beckman TLA 120.2 rotor. The resulting supernatant (S_{Ti8}) was subjected to precipitation of proteins by addition of trichloroacetic acid to 10%, followed by washing of the precipitate in ice-cold 80% (v/v) acetone, SDS-polyacrylamide gel electrophoresis, and immunoblotting. The peroxisome membrane pellet (P_{Ti8}) was resuspended in ice-cold Ti8 buffer and subjected to sodium carbonate extraction (29). After incubation on ice for 45 min with occasional agitation, the samples were centrifuged at 200,000 × g_{av} for 1 h at 4 °C in a Beckman TLA 120.2 rotor. The resulting pellets were resuspended in ice-cold Ti8 buffer. Proteins from both the supernatant and pellet fractions were analyzed by SDS-polyacrylamide gel electrophoresis as described above, and the proteins were transferred to nitrocellulose for immunoblotting. Treatment of peroxisomes with Ti8 buffer quantitatively liberates soluble matrix proteins to the S_{Ti8} fraction (20).

Electron Microscopy—Electron microscopy of whole yeast cells was performed as described elsewhere (28).

Antibodies—Antibodies to proteins synthesized as fusions with the maltose binding protein of *Escherichia coli* were raised in guinea pigs and rabbits (30). Fusions were made by inserting fragments of genes encoding the various proteins in-frame and downstream of the open reading frame encoding the maltose binding protein in the vector pMAL-c2 (New England Biolabs, Beverly, MA), followed by expression in *E. coli*. The individual fusions consisted of a 1648-bp *Eco*RI/*Bam*HI fragment coding for amino acids 49–380 of Pay5p, a 1916-bp *Xho*I/*Sal*I fragment coding for amino acids 11–414 of *Y. lipolytica* peroxisomal thiolase, a 801-bp *Sal*I/*Hin*dIII fragment coding for amino acids 288–555 of *Y. lipolytica* peroxisomal isocitrate lyase, and a 1644-bp *Eco*RV/*Bgl*II fragment encoding amino acids 93–641 of *S. cerevisiae* peroxisomal acyl-CoA oxidase. Anti-SKL and anti-Pay32p antibodies have been described (20). Antibodies to *S. cerevisiae* malate synthase were kindly provided by Dr. A. Hartig (Institute of Biochemistry and Molecular Cell Biology, Vienna, Austria).

Transfection and Immunofluorescence Microscopy of Chinese Hamster Ovary Cells—Chinese hamster ovary (CHO) cells were maintained in F-12 medium (Life Technologies, Inc.) containing 10% (v/v) fetal calf serum. Transfections were performed as described previously (25). Transfections contained 1.7 μg of the expression plasmid pSG5-*PAY5* (parental plasmid = pSG5 (31)) and 0.3 μg of pRSVCAT expressing bacterial chloramphenicol acetyl transferase (32), or 2 μg of pRSVCAT alone. Immunofluorescence microscopy was performed as described elsewhere (33).

Analytical Procedures—Enzymatic activities of catalase, 3-hydroxyacyl-CoA dehydrogenase, and cytochrome *c* oxidase (20) were determined by established methods. Quantitation of immunoblots was performed as described previously (20). Northern blot analysis was performed as described previously (30).

RESULTS

The pay5-1 Mutant Shows Abnormal Peroxisome Morphology—The *pay5-1* mutant was unable to use oleic acid as a carbon source (Fig. 1) and was impaired in the targeting of a number of peroxisomal matrix proteins (see below). Electron micrographs of wild-type *E122* cells grown in oleic acid-containing medium (YPBO = 0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 1% Brij 35, 1% (w/v) oleic acid) showed numerous large, round peroxisomes well separated from one another and surrounded by single unit membranes (Fig. 2*A*). In contrast, *pay5-1* cells showed irregular structures consisting of vesicular elements surrounded by closely apposed, multiple unit membranes (Fig. 2*B*).

Isolation and Characterization of the PAY5 Gene—The *PAY5* gene was isolated from a *Y. lipolytica* genomic DNA library by

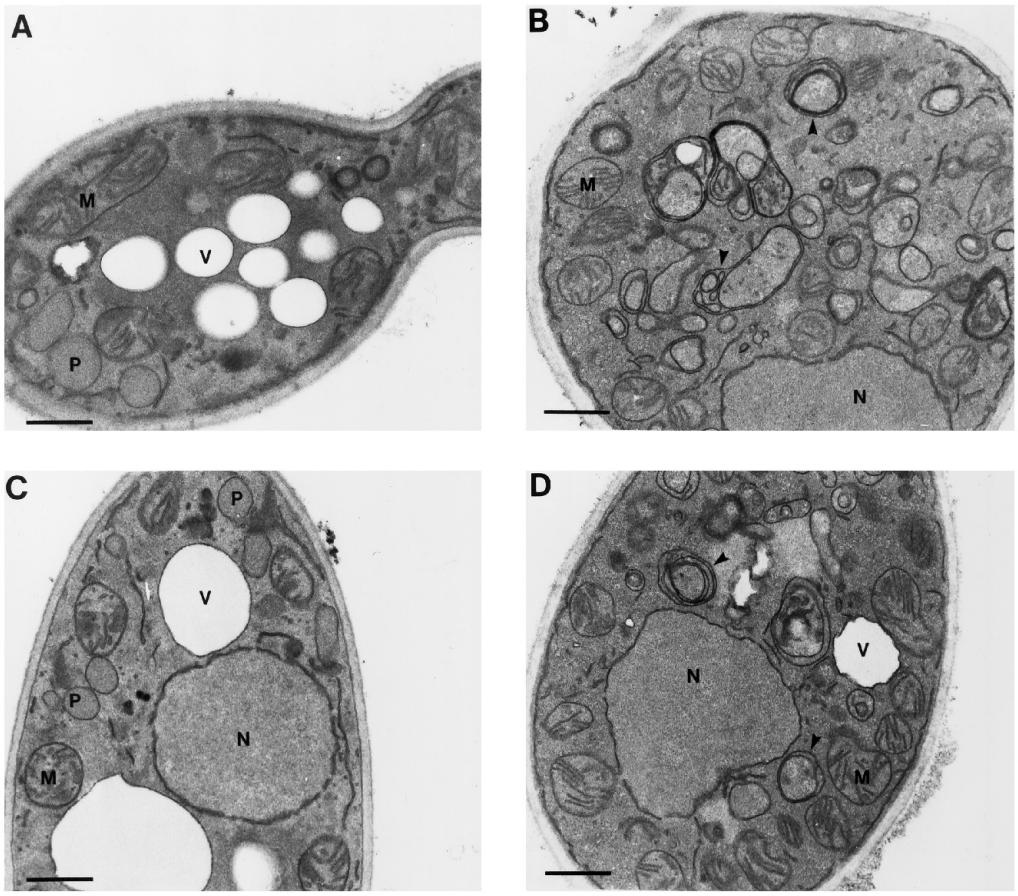

FIG. 2. **Ultrastructure of the wild-type *E122* and *pay5* mutant strains.** The *E122* (*A*), *pay5–1* (*B*), *PAY5* (*C*), and *P5KO-8A* (*D*) strains were grown for 10 h in YEPD (1% yeast extract, 2% peptone, 2% glucose) medium, transferred to YPBO (0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 1.0% Brij 35, 1.0% (w/v) oleic acid), and grown for an additional 8 h. Cells were fixed in 1.5% $KMnO_4$ and processed for electron microscopy. *Arrowheads* indicate vesicular elements surrounded by multiple unit membranes. *P*, peroxisome; *M*, mitochondrion; *N*, nucleus; *V*, vacuole. *Bar* = 0.5 μm.

functional complementation of the mutation in *pay5-1* cells. Of the 5×10^3 transformants screened, one strain, *PAY5*, had restored growth on oleic acid (Fig. 1) and showed wild-type peroxisomal morphology (Fig. 2*C*). The *PAY5* strain carried the plasmid p5S11, which contains an ~5.6-kbp insert of *Y. lipolytica* DNA (Fig. 3*A*). Subcloning of fragments from this insert localized the ability to functionally complement the *pay5-1* mutation to an ~2.0-kbp *Hin*dIII/*Bam*HI fragment. Sequencing within this fragment revealed an open reading frame of 1134 nucleotides, the putative *PAY5* gene, encoding a 380 amino acid protein, Pay5p, with a predicted molecular mass of 41,720 Da (Fig. 3*B*).

The putative *PAY5* gene was disrupted by integration of the *Y. lipolytica LEU2* gene to make the strains *P5KO-8A* and *P5KO-9B* in the A and B mating types, respectively (Table I). These strains were unable to grow on oleic acid (Fig. 1) and had the same peroxisomal morphology (Fig. 2*D*) and peroxisomal protein targeting defects (see below) as the original *pay5-1* mutant. The diploid strains *D5-OB*, *D5-A9*, and *D5–8B* (Table I) could grow on oleic acid (Fig. 1), demonstrating the recessive nature of the *pay5-1* mutation. The diploid strain *D5-O9* from the mating of *pay5-1* and *P5KO-9B* (Table I) was unable to grow on oleic acid (Fig. 1), indicating that the authentic *PAY5* gene had been cloned.

Northern blot analysis (Fig. 4) showed a large induction in the level of mRNA encoding Pay5p 4 h after shifting wild-type *E122* cells from growth in glucose-containing medium to growth in oleic acid-containing medium. The levels of mRNA encoding Pay5p were lower than those encoding thiolase, a peroxisomal matrix protein (Fig. 4).

Pay5p Is a Y. lipolytica Homologue of Mammalian PAF-1—A C_3HC_4- RING-finger motif (24) is found toward the carboxyl terminus of Pay5p (Fig. 3*B*, *underlined*). A search of protein data bases using the GENEINFO (R) BLAST Network Service (Blaster) of the National Center for Biotechnology Information identified a number of proteins with homology to Pay5p within the RING-finger motif. Pay5p also showed significant sequence homology outside this motif to four proteins: human, rat and CHO PAF-1 proteins and to fungal CAR-1 protein. Pay5p exhibits 32, 32, and 33% identity with human, rat, and CHO PAF-1, respectively. All PAF-1 proteins are integral to the peroxisomal membrane (25, 26, 34). Mutations in PAF-1 affect peroxisome assembly. Mutation in human PAF-1 results in Zellweger (cerebrohepatorenal) syndrome (26). Pay5p shows 43% identity to the CAR-1 protein of the fungus *Podospora anserina* (35), which in turn shows 27% identity to all PAF-1 proteins. Mutations in the CAR-1 protein lead to peroxisome and sexual karyogamy defects (35). Alignment of Pay5p with human PAF-1 and CAR-1 proteins (Fig. 5) shows several regions of strong homology outside the C_3HC_4 domain, including the amino-terminal portions of the proteins and a stretch of 39 amino acids (amino acids 195–234 of Pay5p) encompassing hydrophobic segments within all three proteins. Both regions of strong homology are known to be essential for CHO PAF-1 function (34).

Pay5p Is a Peroxisomal Integral Membrane Protein—Hy-

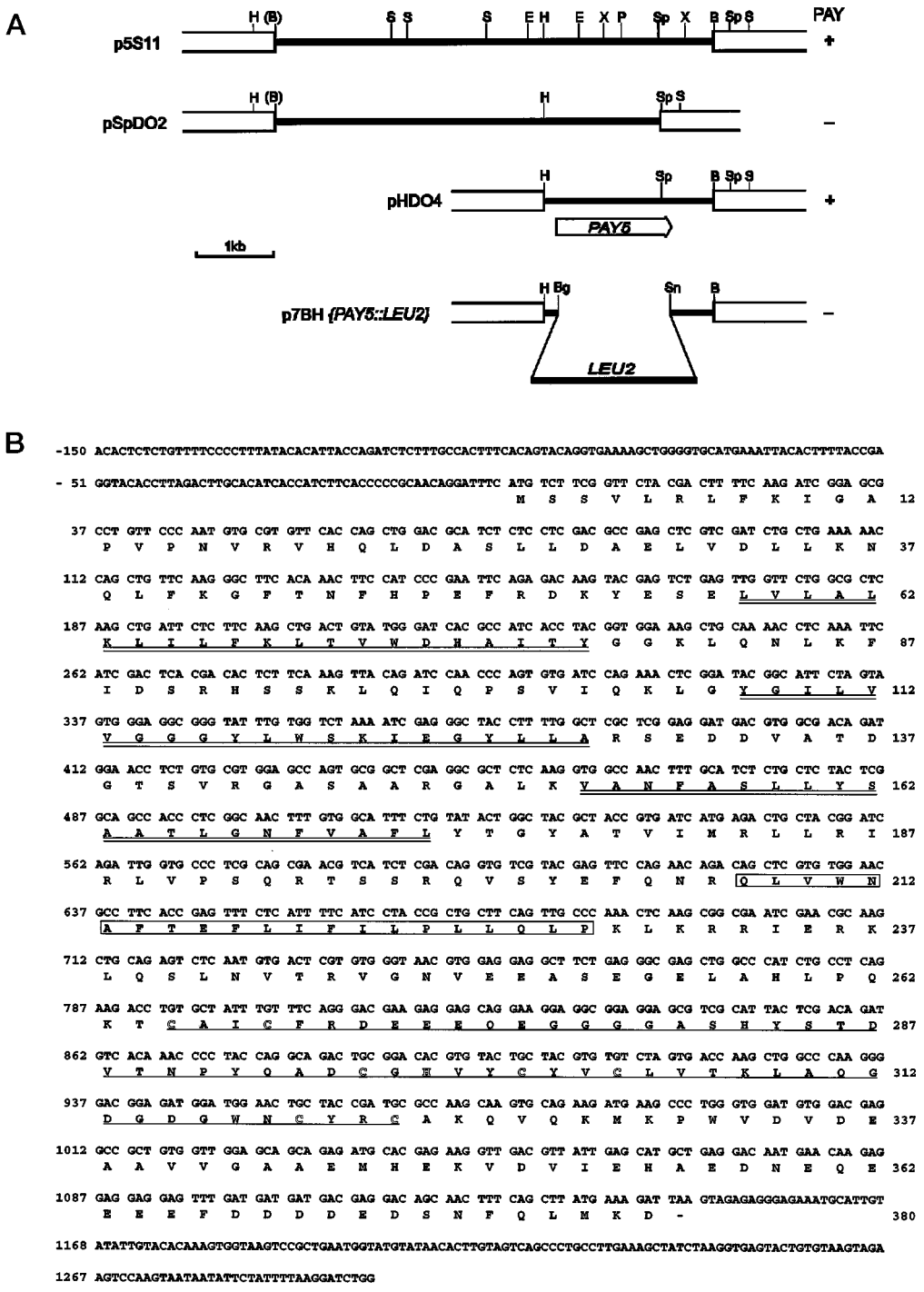

FIG. 3. **Cloning and analysis of the *PAY5* gene.** *A*, complementing activity of inserts, restriction map analysis, and targeted gene disruption strategy for the *PAY5* gene. *Solid lines*, *Y. lipolytica* genomic DNA; *open boxes*, vector DNA. The open reading frame of the *PAY5* gene is indicated by the *wide arrow*. The (+) symbol denotes the ability of an insert to confer growth on oleic acid to *pay5-1*. The (−) symbol denotes the inability of an insert to confer growth on oleic acid to *pay5-1*. *B*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pst*I; *S*, *Sal*I; *Sn*, *Sna*BI; *Sp*, *Sph*I; *X*, *Xho*I. *B*, nucleotide sequence of the *PAY5* gene and deduced amino acid sequence of Pay5p. *Underlined residues*, C_3HC_4-RING-finger motif; *boxed residues*, predicted membrane-spanning α-helix; *doubly underlined residues*, predicted membrane-associated helices. The nucleotide sequence reported in this study has been submitted to GenBank™ with accession no. U43081.

dropathy analysis (36) showed Pay5p to be hydrophobic overall and most likely a membrane protein (data not shown). Based on algorithms predicting membrane-spanning regions of proteins (see Ref. 30 and references therein), Pay5p is predicted to contain one membrane-spanning α-helix (Fig. 3*B*, *boxed residues*) and three membrane-associated helices (Fig. 3*B*, *doubly underlined*). Immunoblot analysis of subcellular fractions and peroxisomes purified from YPBO-grown wild-type cells, with anti-Pay5p antibodies, showed Pay5p to be localized to peroxisomes (Fig. 6, *lane PX*). Pay5p was absent in a 20KgS fraction enriched for cytosol (Fig. 6, *lane S*). Lysis of peroxisomes with Ti8 buffer or treatment with sodium carbonate followed by high-speed centrifugation showed Pay5p to be localized exclusively to the pellet fractions (Fig. 6, compare *lanes* P_{Ti8} and S_{Ti8}, and *lanes* P_{CO3} and S_{CO3}), consistent with Pay5p being integral to the peroxisomal membrane. Pay5p was not found in either the 20KgS (*lane S*) or 20KgP (*lane P*) from the *pay5-1* mutant.

Pay5p was targeted to mammalian peroxisomes *in vivo*. Immunofluorescence microscopy showed that Pay5p localized to peroxisomes and colocalized with anti-SKL-reactive proteins in CHO cells transfected with the *PAY5* gene (Fig. 7). Therefore, the mechanism of targeting integral membrane proteins to peroxisomes appears to have been conserved between yeast and mammalian cells.

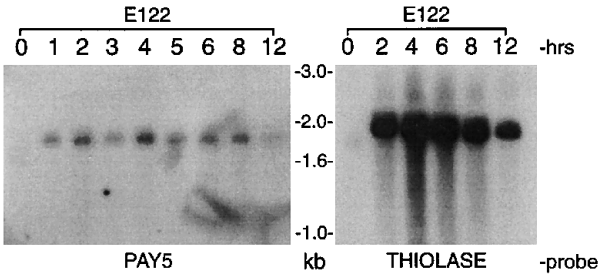

FIG. 4. **Pay5p mRNA and peroxisomal thiolase mRNA are induced by growth of *Y. lipolytica* in oleic acid.** Total RNA was isolated from strain *E122* grown in glucose medium (YEPD, 0 h) and after transfer to oleic acid medium (YPBO) for different periods of time (numbers at the top of the figure indicate hours). 10 μg of RNA from each time point was analyzed on a formaldehyde-agarose gel and transferred to nitrocellulose. The blots were hybridized with radiolabeled probes specific for the *PAY5* gene and the gene encoding peroxisomal thiolase. The numbers between the two panels indicate the electrophoretic migrations of DNA markers (in kbp). Exposure time was 5 d for the Pay5p mRNA and 20 h for the thiolase mRNA.

Peroxisomal Proteins Mislocalize to the Cytosol to Varying Extents in Pay5 Mutants—Mutations in the *PAY5* gene do not affect the synthesis of peroxisomal proteins. When wild-type, *pay5-1* and *P5KO-8A* strains were grown in YEPD for 10 h and then shifted to YPBO for a further 8 h, the levels of peroxisomal proteins were greatly induced (data not shown) and reached steady state in all three strains. No significant differences in the steady state levels of all peroxisomal proteins analyzed were found in the three strains (Fig. 8, *top panel*). However, the distribution of these proteins between the 20KgP and the 20KgS was altered in both mutants as compared to the wild-type strain. While in wild-type cells from 82 to 98% of all peroxisomal proteins analyzed were associated with the 20KgP, a significant fraction of these proteins was mislocalized to the 20KgS in the mutant strains (Fig. 8, middle and bottom panels, respectively). Furthermore, the extent of mislocalization to the cytosol in the *pay5* mutants varied for different peroxisomal proteins. The mislocalization of isocitrate lyase and catalase (up to 91% in the 20KgS) was more pronounced than the mislocalization of thiolase, 3-hydroxyacyl-CoA dehydrogenase and Pay32p (up to 67%) and, especially, of malate synthase, acyl-CoA oxidase and a 62 kDa anti-SKL-reactive protein (up to 46%).

DISCUSSION

Here we report the isolation of *pay5* strains, their morphological and initial biochemical characterizations, the cloning

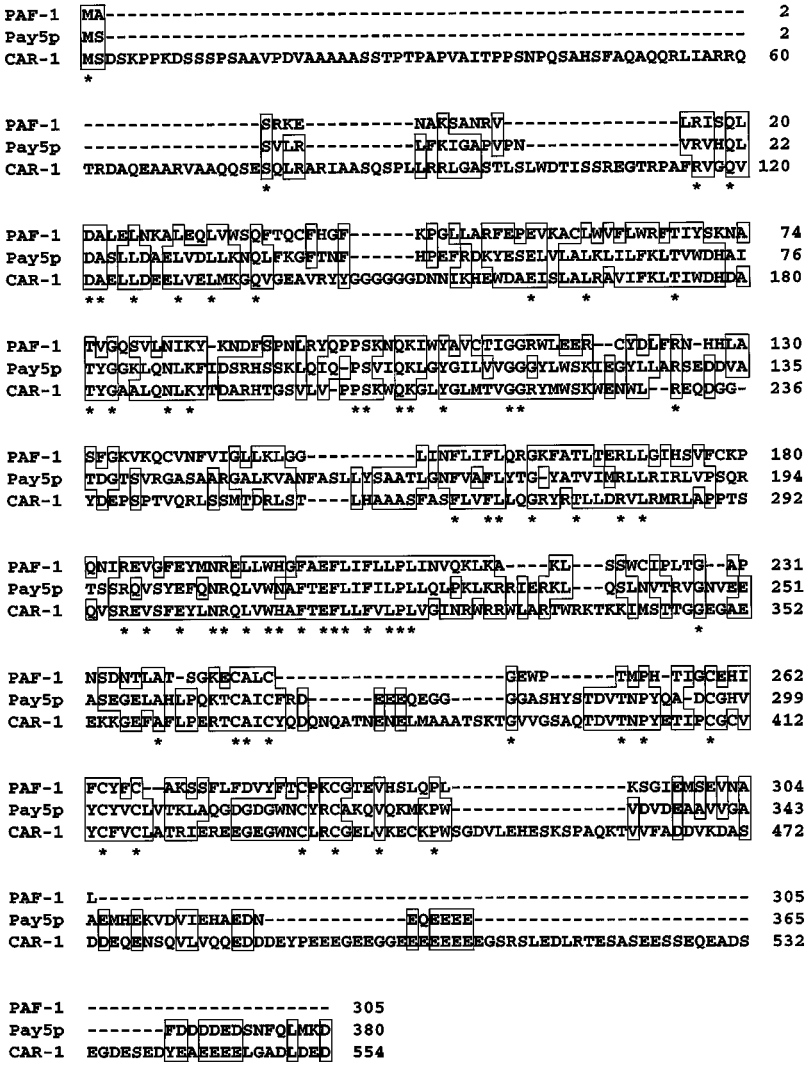

FIG. 5. **Pay5p is a homologue of mammalian PAF-1.** The amino acid sequence of Pay5p was aligned with the amino acid sequences of human PAF-1 and fungal CAR-1. Identical and similar residues are *boxed*. Residues identical in all three sequences are indicated by an *asterisk*. Similarity rules: I = L = V = M; A = S = T; F = Y = W; K = R; D = E; Q = N.

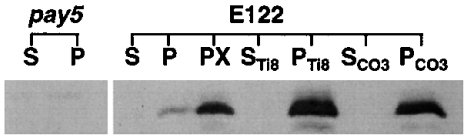

FIG. 6. **Pay5p is a peroxisomal integral membrane protein.** Immunoblot analysis of 20KgS (*S*) and 20KgP (*P*) subcellular fractions, whole peroxisomes (*PX*) and peroxisomal subfractions (S_{Ti8}, P_{Ti8}, S_{CO3}, and P_{CO3}) of the wild-type *E122* and *pay5-1* mutant strains, with anti-Pay5p antibodies. Equal portions (0.5% of total fraction volume) of the 20KgS and 20KgP fractions were analyzed. Purified peroxisomes (30 μg of protein) were lysed with Ti8 buffer and subjected to centrifugation to yield a 245,000 $\times$ g_{max} supernatant (S_{Ti8}) and pellet (P_{Ti8}). S_{CO3} and P_{CO3} correspond to the 245,000 $\times$ g_{max} supernatant and pellet, respectively, recovered after treatment of the P_{Ti8} with 0.1 M Na_2CO_3 (pH 11).

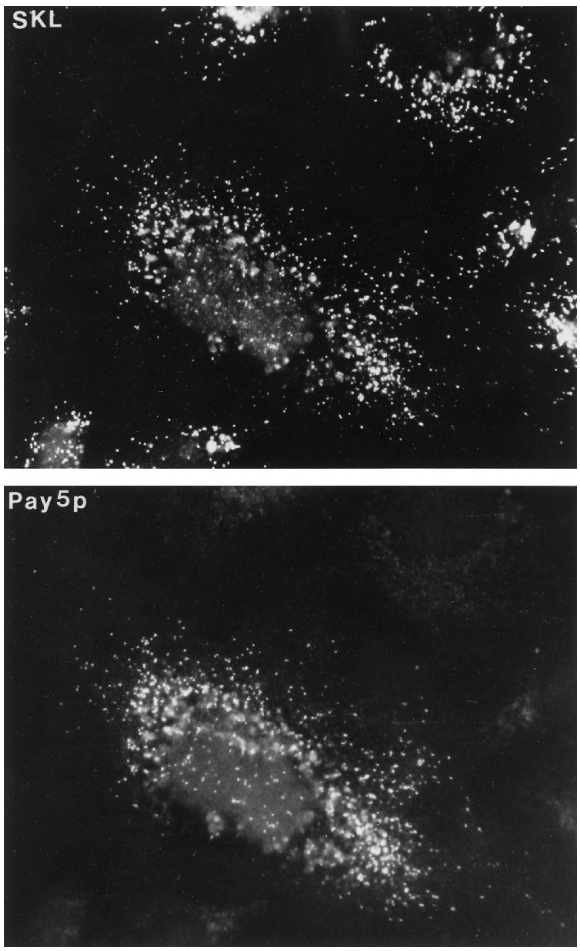

FIG. 7. **Pay5p is targeted to peroxisomes of CHO cells.** Double labeling, indirect immunofluorescence analysis of the CHO wild-type cell line CHO-K1 transfected with the plasmid pSG5-*PAY5* expressing the *PAY5* gene. Primary antibodies were rabbit anti-SKL (*SKL*) and guinea pig anti-Pay5p (*Pay5p*). Primary antibodies were detected with fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated donkey anti-guinea pig IgG secondary antibodies. The transfected cell in the center of the field is decorated by both anti-SKL and anti-Pay5p antibodies. Cells surrounding this central cell are not transfected and do not show decoration by anti-Pay5p antibodies. However, these cells are still decorated by anti-SKL antibodies.

and sequencing of the *PAY5* gene, and the identification and characterization of the *PAY5* gene product, Pay5p.

Pay5 mutant strains cannot assemble functional peroxisomes. They are unable to grow using oleic acid as the carbon source, and, under conditions of peroxisome induction, they accumulate vesicular structures that are surrounded by multiple membranes. *Pay5* mutant strains can apparently import

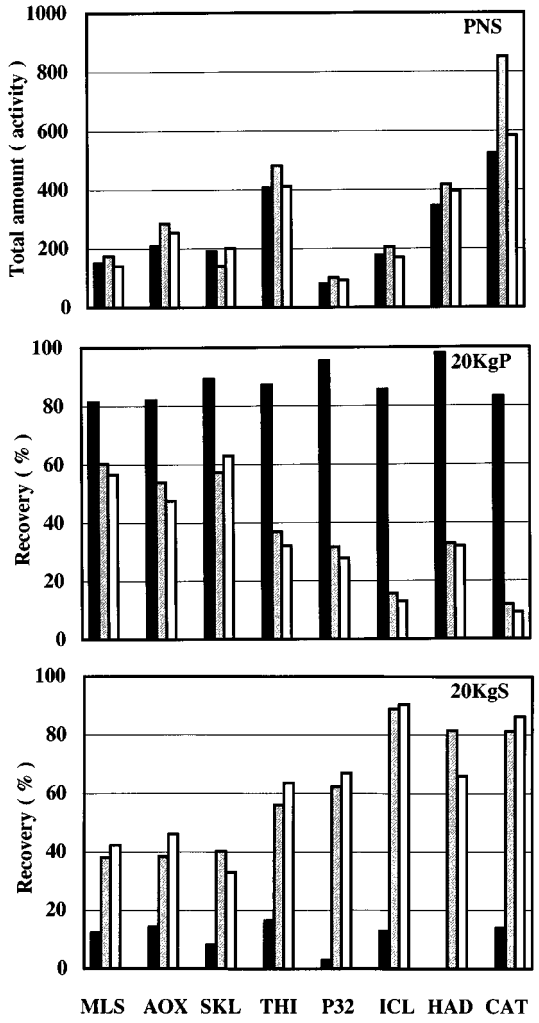

FIG. 8. **Peroxisomal proteins are synthesized normally but are mislocalized to the cytosol to varying extents in *pay5* mutants.** Wild-type *E122* (*solid bar*), *pay5-1* (*stippled bar*), and *P5KO-8A* (*open bar*) strains were grown as described under "Materials and Methods." Cells were subjected to subcellular fractionation. The total enzymatic activities of catalase (*CAT*) and 3-hydroxyacyl-CoA dehydrogenase (*HAD*) contained in 10 mg of protein of postnuclear supernatant (*PNS*) are expressed either in units (CAT) or in milliunits $\times$ 10 (HAD) (*top panel*). The percentage of total enzymatic activity of CAT and HAD recovered in the 20KgP (*middle panel*) and 20KgS (*bottom panel*) are shown. Equal fractions (0.2% of the total volume) of the PNS, 20KgP, and 20KgS were also analyzed by immunoblotting with anti-malate synthase (*MLS*), anti-acyl-CoA oxidase (*AOX*), anti-SKL (*SKL*), anti-thiolase (*THI*), anti-Pay32p (*P32*), and anti-isocitrate lyase (*ICL*) antibodies. Immunoblots were quantitated by densitometry (arbitrary units). SKL, quantitation was performed for the 62-kDa anti-SKL-reactive polypeptide.

some, but not all, peroxisomal matrix proteins. Indeed, the import of acyl-CoA oxidase and malate synthase in *pay5* mutants is only slightly less than that observed in the wild-type strain. In contrast, the import of catalase and isocitrate lyase is much less in *pay5* mutants *vis-à-vis* that observed in wild-type cells. These data suggest that components involved in the import of catalase and isocitrate lyase to peroxisomes are selectively affected by mutations in Pay5p. Furthermore, *pay5* mutants are not affected selectively in the import of either PTS1 or PTS2 proteins. Both a 62-kDa SKL (PTS1)-containing polypeptide and thiolase, which contains a PTS2 motif (37), are imported with comparable efficiencies in *pay5* mutants. On the other hand, *pay5* mutations strongly affect the import of isocitrate lyase, which is known to contain a PTS1 motif (38). One possible explanation for the observed differences in import of

the two PTS1-containing proteins in *pay5* mutants is that one protein could be imported into the peroxisome by an independent alternative (redundant) PTS, as has been reported for *S. cerevisiae* catalase A (39) and *H. polymorpha* Per1p (40).

What structural features of Pay5p may be important for its role in peroxisome assembly? Pay5p belongs to the C_3HC_4-RING-finger protein superfamily. This family encompasses a diverse group of proteins originally thought to mediate transcription through binding to DNA via the C_3HC_4 motif (24). Recently, this domain has been found in proteins involved in organelle assembly, *e.g.* Pep3/Vsp18p (41, 42) and Pep5/Vsp11p/End1 (43, 44) of *S. cerevisiae*, which are involved in vacuole biogenesis, and *H. polymorpha* Per8p (45), *S. cerevisiae* Pas4p (46), and *P. pastoris* Pas7p (47), which are required for peroxisome biogenesis in yeast. All of these proteins, including Pay5p, have been localized to the membranes of their respective organelles and, accordingly, DNA binding is not readily possible. It is also noteworthy that the C_3HC_4 domain is always located near to the carboxyl termini of these proteins. Therefore, a subfamily of RING-finger proteins can be defined that contains proteins which have C_3HC_4 domains at their carboxyl termini and which have roles in organelle biogenesis. The C_3HC_4 domains may act to stabilize tertiary protein structure (48), protein-protein interactions (49, 50), or protein-lipid interactions (51).

Pay5p shows limited homology to most C_3HC_4-containing proteins involved in organelle biogenesis outside of the RING-finger domain, with the exception of fungal CAR-1 and mammalian PAF-1 proteins. Interestingly, CAR-1 was discovered by complementation of a karyogamy (nuclear fusion) mutant of the fungus *P. anserina* (35). The CAR-1 protein was localized to peroxisomal membranes and was essential for fungal peroxisome development (35), as is the case for Pay5p in *Y. lipolytica* and for PAF-1 in mammalian cells. We observed that *pay5* mutant strains fail to assemble peroxisomes delimited by a single unit membrane. This observation, combined with results from studies of other RING-finger proteins required for peroxisome biogenesis (35, 45, 47), suggests a role for Pay5p in the assembly of the peroxisomal membrane.

Lastly, while the evolutionary conservation of the mechanisms of import of peroxisomal matrix proteins has been well established (1–4), this study provides evidence that the mechanism of targeting integral membrane proteins to peroxisomes has also been conserved between yeast and mammalian cells.

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