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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 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, PaySp, of 380 amino acids (41,720 Da). PaySp 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. PaySp 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 organelar locations. Accordingly, eukaryotic cells have developed mechanisms for recognizing newly made organelar proteins and for targeting them to their correct destinations.

In the case of peroxisomes, two types of peroxisomal targeting signals (PTS) act to direct proteins to the peroxisomal 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 terminus 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), Hanzonula 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 PaySp, a peroxisomal integral membrane protein that contains a C_3HC_4-RING-finger domain (24). PaySp 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 pNA445 (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-
TABLE I

Y. lipolytica strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>E122</td>
<td>MATA,ura3-302,leu2-270,lys8-11</td>
</tr>
<tr>
<td>22301-3</td>
<td>MATA,ura3-302,leu2-270,his1</td>
</tr>
<tr>
<td>pay5-1</td>
<td>MATA,ura3-302,leu2-270,lys8-11,pay5-1</td>
</tr>
<tr>
<td>PAY5</td>
<td>MATA,ura3-302,leu2-270,lys8-11,pay5-1,pBH7(LEU2)</td>
</tr>
<tr>
<td>P5KO-9A</td>
<td>MATA,ura3-302,leu2-270,lys8-11,pay5-1,pAY5-1,LEU2</td>
</tr>
<tr>
<td>P5KO-9B</td>
<td>MATA,ura3-302,leu2-270,his1,pay5-1,LEU2</td>
</tr>
<tr>
<td>DS-OB</td>
<td>MATA/MATB,ura3-302,leu2-270,leu2-270,lys8-11/+,-,his1,pay5-1/+</td>
</tr>
<tr>
<td>DS-09</td>
<td>MATA/MATB,ura3-302,leu2-270,leu2-270,lys8-11/+,-,his1,+/pay5-1,LEU2</td>
</tr>
<tr>
<td>DS-A9</td>
<td>MATA/MATB,ura3-302,leu2-270,leu2-270,lys8-11/+,-,his1,+/pay5-1,LEU2</td>
</tr>
<tr>
<td>DS-88</td>
<td>MATA/MATB,ura3-302,leu2-270,leu2-270,lys8-11/+,-,his1,+/pay5-1,LEU2</td>
</tr>
<tr>
<td>DS-89</td>
<td>MATA/MATB,ura3-302,leu2-270,leu2-270,lys8-11/+,-,his1,+/pay5-1,LEU2</td>
</tr>
<tr>
<td>D1-AB</td>
<td>MATA/MATB,ura3-302,leu2-270,leu2-270,lys8-11/+,-,his1,+/pay5-1,LEU2</td>
</tr>
</tbody>
</table>

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 PAYS is compared to that of the wild-type strain E122, the original pay5-1 mutant, the genotype disruption strains P5KO-BA and P5KO-9B, and the diploid strains DS-A9, DS-09, D1-AB, DS-89, DS-OB, and DS-88. 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.

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 EcoRI/BamHI fragment coding for amino acids 49–380 of Pay5p, a 1916-bp XhoI/SalI fragment coding for amino acids 11–414 of Y. lipolytica peroxisomal thiolase, a 801-bp SalI/HindIII fragment coding for amino acids 288–555 of Y. lipolytica peroxisomal isocitrate lyase, and a 1644-bp EcoRV/BglII 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 pRSV CAT expressing bacterial chloramphenicol acetyl transferase (32), or 2 μg of pHASV CAT 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% K2HPO4, 0.5% KH2PO4, 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. 2A). In contrast, pay5-1 cells showed irregular structures consisting of vesicular elements surrounded by closely apposed, multiple unit membranes (Fig. 2B).
functional complementation of the mutation in pay5-1 cells. Of the $5 \times 10^5$ transformants screened, one strain, PAY5, had restored growth on oleic acid (Fig. 1) and showed wild-type peroxisomal morphology (Fig. 2C). The PAY5 strain carried the plasmid p5S11, which contains an $\approx$5.6-kbp insert of Y. lipolytica DNA (Fig. 3A). Subcloning of fragments from this insert localized the ability to functionally complement the pay5-1 mutation to an $\approx$2.0-kbp HindIII/BamHI 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. 3B).

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. 2D) 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 C3HC4 RING-finger motif (24) is found toward the carboxyl terminus of Pay5p (Fig. 3B, 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 C3HC4 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, nucleotide sequence of the PAY5 gene and deduced amino acid sequence of Pay5p. Underlined residues, C3HC4-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.

A drophath 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. 3B, boxed residues) and three membrane-associated helices (Fig. 3B, 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 peroxi-
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.

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 doing
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 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 as compared to 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, 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. One possible explanation for the observed differences in import of 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 as compared to 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, 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. 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 \textit{S. cerevisiae} catalase A (39) and \textit{H. polymorpha} Perp1 (40). What structural features of Pay5p may be important for its role in peroxisome assembly? Pay5p belongs to the \textit{C}_{4}HC_{4}-RING-finger protein superfamily. This family encompasses a diverse group of proteins originally thought to mediate transcription through binding to DNA via the \textit{C}_{4}HC_{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 \textit{S.cerevisiae}, which are involved in vacuole biogenesis, and \textit{H. polymorpha} Perp5 (45). \textit{S.cerevisiae} Pas5p (46), and \textit{P. pastoris} and Gould, S. J. (1993) J. Biol. Chem. 268, 607–613.


