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The *Pichia pastoris* PER6 Gene Product Is a Peroxisomal Integral Membrane Protein Essential for Peroxisome Biogenesis and Has Sequence Similarity to the Zellweger Syndrome Protein PAF-1

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We report the cloning of *PER6*, a gene essential for peroxisome biogenesis in the methylotrophic yeast *Pichia pastoris*. The *PER6* sequence predicts that its product Per6p is a 52-kDa polypeptide with the cysteine-rich C3HC4 motif. Per6p has significant overall sequence similarity with the human peroxisome assembly factor PAF-1, a protein that is defective in certain patients suffering from the peroxisomal disorder Zellweger syndrome, and with car1, a protein required for peroxisome biogenesis and caryogamy in the filamentous fungus *Podospora anserina*. In addition, the C3HC4 motif and two of the three membrane-spanning segments predicted for Per6p align with the C3HC4 motifs and the two membrane-spanning segments predicted for PAF-1 and car1. Like PAF-1, Per6p is a peroxisomal integral membrane protein. In methanol- or oleic acid-induced cells of *per6* mutants, morphologically recognizable peroxisomes are absent. Instead, peroxisomal remnants are observed. In addition, peroxisomal matrix proteins are synthesized but located in the cytosol. The similarities between *PER6* and *PAF1* in amino acid sequence and biochemical properties, and between mutants defective in their respective genes, suggest that Per6p is the putative yeast homolog of PAF-1.

Peroxisomes are organelles found in virtually all eukaryotic cells and are morphologically characterized by a single membrane surrounding a protein-rich matrix. The biochemical hallmarks of peroxisomes are the presence of catalase and at least one hydrogen peroxide-generating oxidase (8). Their size, abundance, and enzymatic content reflect their specific metabolic function and vary depending upon the organism, cell type, and environmental conditions. Peroxisomes are involved in several essential catabolic and anabolic pathways. For example, in mammalian cells, the organelles are involved in the oxidative degradation of fatty acids (*b*-oxidation), purines, D-amino acids, and piperic acid, as well as in the biosynthesis of ether-linked glycerolipids (including plasmalogens), cholesterol, and bile acids (36, 62). Peroxisomes are indispensable for human survival as demonstrated by the existence of genetic deficiencies in a single peroxisomal enzyme to defects in genes essential for assembly or biogenesis of functional peroxisomes. The most severe disorder, Zellweger syndrome, is characterized by the absence of peroxisomes. Complementation analysis of human Zellweger syndrome cell lines has revealed that mutations in any one of at least nine different genes are responsible for this disorder (53). Three of these genes have been identified. The first gene encodes PMP70, a member of the ATP-binding cassette transporter family (18, 19, 29). The third and most recently discovered gene, *PXR1* or *PTS1R*, was identified by the similarity of its product to the yeast *Pichia pastoris* *PAS8* gene product, a putative import receptor of one class of peroxisomal matrix proteins (11, 42, 69), and by a two-hybrid screen (16).

Since peroxisomes do not contain DNA or ribosomes, proteins destined for the organelle are most likely encoded by nuclear genes. Peroxisomal proteins are synthesized in the cytoplasm and posttranslationally imported into the organelar matrix or surrounding membrane (3, 35). Two distinct classes of peroxisomal targeting sequences responsible for correct delivery of matrix proteins to the organelles have been identified. The first is PTS1, a tripeptide of the sequence SKL (and conservative variants) that is present at the extreme carboxy terminus of many matrix proteins (9). PTS1 functions in animals, plants, and yeasts and thus has been conserved through evolution (22, 23, 57). The second, PTS2, with the consensus sequence RLX,HLQL, was initially identified at the amino terminus of 3-keto-acyl coenzyme A thiolase of mammals (46, 56) and yeast (21) but has recently been identified on watermelon glyoxysomal malate dehydrogenase (20), amine oxidase (15), and Per1p (67) from the yeast *Hansenula polymorpha*. Little is known about targeting of peroxisomal membrane proteins, although a 68-amino-acid internal sequence of PMP47, a peroxisomal integral membrane protein from the yeast *Candida boidinii*, is essential for sorting of this protein (41).

Yeasts have emerged as major model systems to investigate the molecular mechanisms involved in peroxisome biogenesis. In addition to the ease of handling of these organisms and their ability to be manipulated by classical and molecular genetic methods, a major advantage of yeasts is that the requirement for peroxisomes for their viability is conditional and can be manipulated by the growth environment (64). Thus, peroxisome biogenesis mutants of yeasts can be readily identified by...
TABLE 1. P. pastoris strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JC100</td>
<td>Wild type</td>
<td>NRR Y-11430</td>
</tr>
<tr>
<td>GS115</td>
<td>his4</td>
<td>6</td>
</tr>
<tr>
<td>GS20/0</td>
<td>his4 arg4</td>
<td>This study</td>
</tr>
<tr>
<td>JC14</td>
<td>per6</td>
<td>This study</td>
</tr>
<tr>
<td>JC16 (per6-1)</td>
<td>per6 his4</td>
<td>This study</td>
</tr>
<tr>
<td>JC214 (per6Δ)</td>
<td>per6 SHIS4 his4 arg4</td>
<td>This study</td>
</tr>
</tbody>
</table>

Cloning and sequence analysis of PER6. To isolate the PER6 gene, the P. pastoris per6-1 mutant JC116 was transformed with a P. pastoris genomic DNA library (37) by the spheroplast transformation method (6). Following selection for histidine prototrophy on YPD plates, the transformants were collected from the plates, pooled, and inoculated in liquid YNM medium at a starting optical density of 0.000 nm (OD600) of 0.3. A culture was observed and the cells were harvested. Plasmids were recovered by transformation of E. coli MC1061 with total DNA extracted from the yeast cells. One vector, named pYT6, was recovered, which, upon transformation of JC116, simultaneously restored histidine prototrophy and methanol growth and was selected for further study. The complementing region was reduced to a 3-kilobase-pair (kb) Clal fragment by testing selected subfragments of the 6.5-kb insert in pYT6 for their ability to complement JC116 for methanol growth. A 1.5-kb Clal-DraI subfragment was subcloned into Clal and DraI- and EcoRl- and EcoRl-digested, bluplaecIs script SK+ (Stratagene, La Jolla, Calif.), and a series of nested deletions were created by limited exonuclease III digestion as described previously (49). Double-stranded DNA sequencing of the resulting subclones was performed by the dideoxy method (50) with Sequenase 2.0 (U.S. Biochemical, Cleveland, Ohio). In addition, several gene-specific 17-bp oligonucleotides were synthesized to complete or confirm the DNA sequence of different regions. For analysis of DNA and amino acid sequences, MacVector software (BIJ, New Haven, Conn.) or PCGENE release 6.3 (IntelliGenetics, Mountain View, Calif.) was used. Sequence alignments were performed with the PALIGN program of PCGENE with the Dayhoff MDM-78 matrix (settings: open gap cost, 150; unit gap cost, 100). The BLAST Network Service of the National Center for Biotechnology Information was used to search for sequence similarities in different protein databases.

**Construction of a P. pastoris disruption strain.** To disrupt the wild-type PER6 gene, a 4.4-kb BamHI fragment from pYT6 was first subcloned into the unique BamHI site of pH322, resulting in a plasmid named pYM8. To construct a disruption mutant JC116, a 1-kb BamHI-Ndel fragment from pYM8 (6) containing the Saccharomyces cerevisiae HIS4 (SHIS4) gene was ligated into BamHI- and Smal-digested pBY5, a derivative of pUC9 in which a SacI site had been converted to a MluI site by insertion of an oligonucleotide linker. The SHIS4 fragment was released from pBY5 with BamHI and MluI and inserted into pRW6 digested with BglII and MluI. This resulted in a deletion of 612 bp of the PER6 open reading frame. The resulting plasmid, named pZ12, was digested by BamHI to yield a 7.9-kb fragment. This fragment was cloned into the unique SalI site of the P. pastoris GS2000 vector and transformed into P. pastoris GS2000. Selected histidine prototrophic transformants were screened for the ability to grow on YNM plates. Methanol utilization-defective (Mut+) strains were examined for correctly targeted genomic integration by Southern blot analysis of chromosomal DNA isolated from several independent Mut+ strains. One per6Δ disruption strain, named JC214, was selected for further studies.

**Preparation of anti-Per6p antibodies.** The carboxy-terminal two-thirds of Per6p was expressed in E. coli as a fusion protein with maltose-binding protein (MBP) with the Protein Fusion and Purification System supplied by New England Biolabs (Beverly, Mass.). To enable subcloning of a 908-bp BglII-HindIII fragment from pYT6 in the MBP reading frame of pMAL-c2, a adapter oligonucleotide (5'-AASCCCGCGGCTCAGGCTCAGC-3') was inserted between the EcoRI and HindIII site of pMAL-c2 to create an in-frame BamHI site. A second adapter oligonucleotide (5'-GAGGGCTTCGCTGCA-3') was then inserted into the PstI site of the vector to create a unique Stul site. The resulting plasmid, pYM8, was digested with BamHI and Stul, and the BamHI-Stul fragment from pYT6 was inserted to produce the MBP-Per6p expression plasmid pMW6. Expression of the MBP-Per6p hybrid protein under control of Pta in E. coli TB1 was induced by addition of 0.3 mM isopropyl-p-D-thiogalactopyranoside to exponentially growing cultures. Purification of the MBP-Per6p protein with amylose resin and DEAE Sepharose CL-6B resin (Pharmacia, Uppsala, Sweden) was performed according to the instructions of New England Biolabs. Purified MBP-Per6p fusion protein was used to immunize rabbits. Per6p-specific antibody preparations were obtained by affinity purification of the antiserum. The crude serum was first passed twice through a CNBr-Sepharose column containing total protein of E. coli TB1 disrupted with pMAL-c2. Subsequently, the flow-through was loaded onto an MBP-Per6p fusion protein column, and then the bound antibodies were eluted with 0.2 M glycine plus 1 mM EDTA (ethylene glycol-bis-[aminomethyl] ether)-N,N,N',N'-tetraacetic acid) (pH 2.4). The eluted fractions were neutralized to pH 7 with 1 M Tris, pooled, and then passed a second time through the MBP-Per6p fusion protein column. After addition of bovine serum albumin to a final concentration of 2 mg/ml, the pooled and neutralized fractions were passed twice through a column containing total protein from the P. pastoris per6Δ disruption mutant JC241. Concentration and dialysis were performed under control of the AOX1p-LUC plasmid (2) in a Centriprep-10 concentrator (Amicon Corp., Beverly, Mass.). The Per6p-specific antibodies were aliquoted and stored at −70°C in phosphate-buffered saline buffer (49).

**Expression of luciferase in yeast strains.** As a reporter protein for PTS1 protein import, the gene encoding firefly luciferase (LUC) was expressed under control of the P. pastoris alcohol oxidase (AOX1) gene promoter (AOXp). The wild-type and per6Δ strains were transformed with the ARG4-based AOX1p-LUC plasmid pAH23 (a gift from S. Subramani, University of California at San Diego). The per6-1 mutant was transformed with pPH107, a plasmid containing the AOX1p-LUC expression cassette from pJAH23 subcloned as a BglII-PvuII

**Materials and Methods.**

**Strains, media, and microbiological techniques.** P. pastoris strains used in this study are listed in Table 1. Shake-flask cultures were grown or induced at 30°C in selective minimal YND, YNM, or YNO medium (0.17% [wt/vol] yeast nitrogen base without amino acids [Difco Laboratories Inc., Detroit, Mich.], supplemented with 0.4% [wt/vol] glucose, 0.5% [vol/vol] methanol, or 0.2% [vol/vol] oleic acid with 0.05% [vol/vol] Tween 40 and 0.05% [wt/wt] yeast extract) or in YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, and 2% [wt/vol] glucose). For peroxisome purifications, cells were grown in continuous cultures at a volume of 2 liters and at 30°C with a Bioflo HI fermentor (New Brunswick Scientific, Edison, N.J.) in a basic mineral medium composed of 0.2 g of MgSO4 ·7H2O per liter, 1 g of KH2PO4 per liter, 0.5 g of yeast extract per liter, 1 ml of a concentrated (1,000×) trace salt solution (see below), and 40 μg of biotin per liter. (NH4)2SO4 (2 g/liter) was used as a nitrogen source. Either 0.5% (vol/vol) methanol or 0.1% (vol/vol) oleic acid plus 0.05% (vol/vol) Tween 40 was used as a carbon and energy source. The tracesalt solution was composed of 4 g of ZnCl2 per liter, 1 g of Na2MoO4 ·H2O per liter, 1.5 g of CaCl2 per liter, 1 g of FeCl3 ·6 H2O per liter, 0.05 g of H3BO3 per liter, 0.3 g of CuSO4 ·5 H2O per liter, and 0.5 g of Na2MoO4 ·2 H2O per liter. For growth of auxotrophic strains, histidine or arginine was added to a final concentration of 50 μg/ml. The sporulation medium (1) and protocols for classical genetic manipulations of P. pastoris have been described previously (38). Transformations of P. pastoris were performed by the spheroplast method (6) or by electroporation (1). Cultivation of Schizosaccharomyces colt (MC1061, DH5α, and TB1) and standard recombiant DNA techniques were performed essentially as described previoulsy (49).
Subcellular fractionation. Wild-type and per6 mutant cells were grown in YPD medium, transferred during logarithmic growth phase by centrifugation into INW, and DDO medium, and then induced at 36 °C for 6 h at 30 °C. Subcellular fractionation of these cells were performed as described previously (38). In addition, subcellular fractions of cells from continuous cultures were performed by the following method. After harvesting by centrifugation (5 min, 6,000 × g) and washing with 0.25 volume of water, the cells were resuspended at an OD600 of 37.5 in 20 mM potassium phosphate buffer (KP, pH 7.5) supplemented with 20 mM β-mercaptoethanol. Following incubation for 15 min at 30 °C, cells were harvested, washed once with KP, alone and once with KP supplemented with 1.2 M sorbitol, and finally suspended at an OD600 of 50 in KP, with 1.2 M sorbitol. Cells were converted to protoplasts by adding 0.8 mg of Zymolase 100T (ICN, Costa Mesa, Calif.) per 500 OD600 units and incubated for 45 to 90 min at 30 °C. All subsequent steps were performed at 4 °C. After harvesting by centrifugation (10 min, 6,000 × g), the protoplasts were gently homogenized at an OD600 of 175 in MES buffer (5 mM 4-morpholinolinesulfonic acid, 1 mM MgCl2, 1 mM EDTA, and 0.1% ethanol) supplemented with 1 M sorbitol, 1 mM phenylmethylsulfonyl fluoride, and NaF; suspension was followed by 10 min of centrifugation and resuspension at 2,500 × g for 10 min. The protoplasts were centrifuged two to four times at 2,500 × g for 10 min until virtually no protoplasts were observed in samples of the supernatant by phase-contrast light microscopy. The organelles in the final supernatant were sedimented at 30,000 × g for 30 min. The resulting organelle pellets, consisting mainly of mitochondria and peroxisomes, and corresponding supernatant were used for biochemical analysis. For purification of peroxisomes, organelle pellets were further fractionated in discontinuous sucrose gradients. The organelle pellet resulting from approximately 3,500 OD600 units of homogenized cells was gently suspended in 4 ml of MES buffer supplemented with 1.2 M sorbitol by a few strokes in a Potter-Elvehjem tissue grinder with a loosely fitting pestle. The organelles were then loaded on top of a sucrose gradient composed of 4 ml of 60%, 5 ml of 50%, 7 ml of 45%, 6 ml of 40%, 3 ml of 35%, and 4 ml of 32% (wt/wt) sucrose in MES buffer and centrifuged at 4 °C for 6 h at 27,000 rpm in a Beckman SW27 Ti rotor. Fractions of approximately 1.2 ml were collected from the bottom of the tube and used for biochemical analysis.

Biochemical methods. Peroxosomal AOX (45) and catalase (CAT) (39), mitochondrial cytochrome c oxidase (12), and cytosolic glyceraldehyde-3-phosphate dehydrogenase (40) activities were assayed at 30 °C by established procedures. Luciferase activity was assayed by the luciferase assay system from Promega (Madison, Wis.) as described previously (45). Protein concentrations were determined with Bio-Rad Laboratories (Hercules, Calif.) or Pierce biocinomic acid (Rockford, Ill.) protein assay kits with bovine serum albumin as a standard. Transfer of proteins onto nitrocellulose after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (34) with the Bio-Rad Mini Trans-Blot Electrotransfer Cell was performed as indicated by the manufacturer. Immunoblotting experiments were performed with the Tropix Western Light Kit (Bedford, Mass.) with specific polyclonal antibodies against per6p (Mouse 2b), AOX, dihydropyruvate synthase (DHAS), or thioredoxin (a gift from W. H. Kunau, Ruhr University, Bochum, Germany).

Electron microscopy. Electron microscopy and immunocytochemistry were performed as described by Waterham et al. (67).

Nucleotide sequence accession number. The data sequence for P. pastoris PER6 are available from EMBL, GenBank, and DDBJ under accession number X98945.

RESULTS

Cloning and sequence analysis of the PER6 gene. The PER6 gene was cloned by functional complementation of the P. pastoris per6-1 mutant JC116 with a P. pastoris genomic DNA library. This mutant was one of a collection of peroxisome-deficient (per) mutants, representing eight different complementation groups isolated in our laboratory (38) (see below for physiological and biochemical characterization of the per6-1 mutant). Library transformants were first selected for histidine prototrophy (His+) and subsequently for restored ability to grow on methanol (Mut+). After transformation of the total DNA extracted from a pool of His+ Mut+ transformants into E. coli, one plasmid, named pYT6, was recovered. pYT6 simultaneously transformed the per6-1 strain to His+ and Mut+ at high frequency, indicating that the plasmid harbored the PER6 gene. The complementing activity in pYT6 was contained within a 6.5-kb genomic DNA insert in the plasmid and was subsequently found to reside within a 3-kb ClaI subfragment. Northern (RNA) blots, with equal amounts of total RNA isolated from glucose- and methanol-grown wild-type cells of P. pastoris and the complementing Clal fragment as probe, revealed a single transcript of approximately 1.4 kb (not shown). The transcript was present at a higher level in methanol-grown cells relative to glucose-grown cells as expected for a PER gene transcript since peroxisomes are strongly induced by methanol in P. pastoris.

The DNA sequence of a major portion of the Clal fragment revealed one open reading frame of 1,383 bp encoding a polypeptide of 461 amino acids with a calculated molecular mass of 52 kDa (Fig. 1A). Hydropathy analysis indicated that the PER6 gene product Per6p possesses several potential membrane-spanning segments (Fig. 1B) (33). On the basis of the algorithm of Klein et al. (30), three membrane-spanning regions were predicted (Fig. 1A; residues 108 to 124, 172 to 189, and 226 to 242) of which one (residues 172 to 189) meets the requirements of a transmembrane α-helix according to the algorithm of Rao and Argos (47). These predictions suggest that Per6p may be an integral membrane protein. The carboxy terminus of Per6p is hydrophilic because of the presence of many aspartic and glutamic acid residues in this region. Overall, the amino acid sequence of Per6p contains more positively charged residues (67) than negatively charged residues (45), resulting in an acidic protein with an isoelectric point of 4.6.

The amino acid sequence of Per6p contains the cysteine-rich C3HC4 motif in the carboxy-terminal half (Fig. 1A; residues 281 to 351). Interestingly, such a C3HC4 motif has been found in several proteins that are essential for peroxisome biogenesis in both yeasts and mammals (see Discussion). A search of protein databases revealed significant overall amino acid sequence similarity between Per6p and PAF-1, a peroxisomal membrane protein essential for human peroxisome biogenesis (Fig. 2; 29% identity, 46% similarity [54]) and its rodent homologs (rat, 26% identity, 44% similarity [60]; Chinese hamster, 28% identity, 46% similarity [61]). Significance was also found with car1 from the filamentous fungus Podospora anserina, a protein required for peroxisome biogenesis and caryogamy and a proposed homolog of the mammalian PAF-1 proteins (Fig. 2; 27% identity, 44% similarity [2]). In addition to the overall similarity in sequence, the C3HC4 motif and two of the three predicted membrane-spanning segments of Per6p align with the C3HC4 motifs and the two membrane-spanning segments predicted for PAF-1 and car1 (Fig. 2; the cysteine-rich motif of car1 has a cysteine substituted for the histidine). Sequence similarity was also observed between Per6p and the unpublished S. cerevisiae CRT1 gene product, Crt1p (29% identity, 42% similarity; GenBank accession no. M86538). Finally, the carboxy terminus of Per6p showed sequence similarity with aspartate- and glutamate-rich domains of numerous proteins. However, this similarity seemed nonspecific since it was strictly limited to the acidic residues in these regions and was not regarded as significant. The acidic carboxy terminus of Per6p is not present in PAF-1 but is observed in car1. In addition, car1 has an amino-terminal extension which is not present in either PAF-1 or Per6p (Fig. 2).

Construction of a PER6 disruption mutant. To confirm that the identified open reading frame is the PER6 gene, a gene disruption was performed by the gene replacement method (48). For the replacement, a plasmid was constructed, one in which 612 bp of the PER6 coding sequence (nucleotides 489 to 1,100 encoding amino acid residues 163 to 367 in Fig. 1A) was replaced by a fragment containing the S. cerevisiae HIS4
The per6D::SHIS4 allele was released on a linear DNA fragment and transformed into P. pastoris GS200 (his4 arg4). Southern blot analysis of total genomic DNA isolated from a randomly selected His<sup>+</sup> transformant that was unable to grow on methanol indicated a correctly targeted chromosomal integration (Fig. 3B). The per6D::SHIS4 strain JC214 (per6D) was crossed with the per6-1 strain JC116, and prototrophic diploid cells were selected on glucose plates and tested for growth on methanol. All were Mut<sup>2</sup>. In addition, after sporulation, approximately 500 spore products were examined, and they also were Mut<sup>2</sup>. Together, these results demonstrate that the per6-1 and the per6D alleles are tightly linked and most probably mutant alleles of the same gene.

Per6p is a peroxisomal integral membrane protein. To determine the subcellular location of Per6p, the contents of methanol- and oleic acid-grown wild-type cells were fractionated by differential centrifugation into an organelle-rich (pellet) fraction, consisting mainly of peroxisomes and mitochondria, and a cytoplasmic (supernatant) fraction (Table 2). In immunoblots with these fractions, affinity-purified antibodies raised against Per6p specifically recognized one major protein species of 58 kDa, as well as a minor species of 45 kDa, that was present in the organelle fraction (Fig. 4A). Neither of these species was observed in similar fractions obtained from methanol- or oleic acid-induced per6D cells (see Fig. 6). Additional experiments revealed that the 58-kDa protein is full-length Per6p and that the lower species is a degradation product (not shown).

To further determine the location of Per6p, the organelle fraction obtained from wild-type cells grown in an oleic acid (SHIS4) gene (Fig. 3A). The per6DAO::SHIS4 allele was released on a linear DNA fragment and transformed into P. pastoris GS200 (his4 arg4). Southern blot analysis of total genomic DNA isolated from a randomly selected His<sup>+</sup> transformant that was unable to grow on methanol indicated a correctly targeted chromosomal integration (Fig. 3B). The per6DAO::SHIS4 strain JC214 (per6Δ) was crossed with the per6-1 strain JC116, and prototrophic diploid cells were selected on glucose plates and tested for growth on methanol. All were Mut<sup>2</sup>. In addition, after sporulation, approximately 500 spore products were examined, and they also were Mut<sup>2</sup>. Together, these results demonstrate that the per6-1 and the per6Δ alleles are tightly linked and most probably mutant alleles of the same gene.

Per6p is a peroxisomal integral membrane protein. To determine the subcellular location of Per6p, the contents of methanol- and oleic acid-grown wild-type cells were fractionated by differential centrifugation into an organelle-rich (pellet) fraction, consisting mainly of peroxisomes and mitochondria, and a cytoplasmic (supernatant) fraction (Table 2). In immunoblots with these fractions, affinity-purified antibodies raised against Per6p specifically recognized one major protein species of 58 kDa, as well as a minor species of 45 kDa, that was present in the organelle fraction (Fig. 4A). Neither of these species was observed in similar fractions obtained from methanol- or oleic acid-induced per6Δ cells (see Fig. 6). Additional experiments revealed that the 58-kDa protein is full-length Per6p and that the lower species is a degradation product (not shown).

To further determine the location of Per6p, the organelle fraction obtained from wild-type cells grown in an oleic acid-
per6 mutants are impaired in peroxisomal matrix protein import. To gain insight into a possible function of Per6p, the physiological and biochemical properties of the chemically induced per6-1 mutant JC116 and the constructed per6Δ mutant JC214 were compared with those of wild-type P. pastoris. Both per6 mutants were unable to grow on methanol or oleic acid as carbon source but grew well on glucose, ethanol, or glycerol. In wild-type cells, methanol or oleic acid induced numerous large peroxisomes that contained enzymes required for the metabolism of these substrates (Fig. 5A). However, in induced cells of both per6 mutants, normal peroxisomes were not observed. Instead, in per6-1 cells, clusters of small vesicular structures that are likely to be peroxisomal remnants were induced (Fig. 5B). Similar structures were also observed in per6Δ cells (Fig. 5D).

Additional evidence for the absence of normal peroxisomes in both per6 mutants was obtained from biochemical experiments aimed at determining the location of peroxisomal matrix proteins. Initial experiments indicated that, in cells of both per6 mutants induced on methanol or oleic acid, most peroxisomal enzymes were present. The location of selected enzymes was determined through subcellular fractionation of induced cells into an organelle pellet (consisting mainly of mitochondria and peroxisomes) and a cytosolic supernatant. The three major peroxisomal matrix enzymes induced on methanol—AOX, DHAS, and CAT—are believed to be PTS1 proteins in P. pastoris, like their counterparts in H. polymorpha (10, 25). After subcellular fractionation of methanol-grown wild-type cells of P. pastoris, a large portion of AOX and CAT activities (Table 2) and of DHAS protein (Fig. 6) was present in the organellar pellet, reflecting their peroxisomal location. How-
ever, significant amounts of these proteins were found in the supernatant, a known consequence of leakage and breakage of peroxisomes during fractionation. As controls, mitochondrial cytochrome c oxidase fractionated to the organelle pellet and cytosolic glyceraldehyde 3-phosphate dehydrogenase fractionated to the supernatant. In contrast to wild-type cells, almost all CAT activity and DHAS protein were located in the supernatant fractions of both per6 mutants, indicating a cytosolic location of these proteins (Table 2 and Fig. 6). Immunoblot analysis indicated that, in both per6 strains, substantial amounts of AOX protein were present. Furthermore, the majority of this protein sedimented upon subcellular fractionation (Fig. 6). However, this was most likely not due to proper AOX import but to aggregation of the protein in the cytosol, a frequently observed phenomenon in methanol-induced per mutants of P. pastoris (Fig. 5C) (37). A small amount of AOX activity was present in the per6-1 mutant and located in the organelle fraction. However, in the per6Δ mutant, no AOX activity could be measured. Such low AOX activity levels are observed for most P. pastoris per mutants (37, 38, 66a) and are believed to be due to an inability of AOX subunits to assemble into mature, active octamers in the cytosol. The presence of a small amount of AOX activity in the organelle pellet of per6-1 cells, but not in that of per6Δ cells, suggests that the peroxisomal remnants in per6-1 cells retain residual import function and, therefore, that the per6-1 allele is slightly leaky. Consistent with this notion, a significant amount of normal-sized Per6p is present in the organelle pellets of induced per6-1 cells (Fig. 6).

To confirm the location of PTS1 proteins in the per6 mutants, the mutants were transformed with the gene encoding the prototypical PTS1 protein luciferase under the control of the AOX1 promoter. This promoter is most active in methanol-induced cells but is also expressed at significant levels in oleic acid-induced cells (55). Organelle and cytoplasmic fractions obtained from oleic acid-induced cells were examined for CAT and luciferase activity (Table 2) and for the PTS2 enzyme thiolase (Fig. 6). While CAT, luciferase, and thiolase were mostly present in the organelle pellet from wild-type cells, they were predominantly found in the cytosolic fractions in both per6 mutants. However, small amounts of thiolase and luciferase were also present in the organelle pellet of the per6-1 mutant. The amount found in the pellet could only partly be explained by contamination of the pellet fraction with cytoplasmic material since little of the cytosolic marker enzyme glyceraldehyde 3-phosphate dehydrogenase was present in the pellet. Thus, as with AOX activity in methanol-induced cells, these data suggest that the per6-1 allele is leaky and that small amounts of peroxisomal enzymes are imported into peroxiso-
mal remnants. In contrast, the small amounts of thiolase and luciferase in the organelle pellet of the per6Δ mutant could be explained by cytoplasmic contamination as indicated by the activity of glyceraldehyde 3-phosphate dehydrogenase in the pellet (Table 2).

**DISCUSSION**

This report describes the identification and characterization of *P. pastoris* Per6p, a peroxisomal integral membrane protein essential for the biogenesis of peroxisomes. Although several proteins essential for peroxisome biogenesis in different yeast species have been identified (5, 13, 14, 26–28, 37, 42, 55, 58, 67), only four are integral components of the peroxisomal membrane. These four are Pas3p of *S. cerevisiae* (27), Pay2p of *Yarrowia lipolytica* (13), Per8p of *H. polymorpha* (58), and its *P. pastoris* homolog Pas7p (28). Per6p is only the second peroxisomal integral membrane protein described for *P. pastoris*, although several peroxisomal membrane-associated proteins have been reported in this yeast. These proteins include Pas8p, the putative PTS1
transporter proteins Pxa1p and ALDp, which are 28% identical (43, 52).

The car1 gene was cloned by complementation of a caryogamy (nuclear fusion) mutant of the filamentous fungus P. anserina and is required for peroxisome biogenesis (2). In contrast to car1 mutants, P. pastoris per6 mutants do not display sexual cycle defects. In crosses between strains with different per6Δ alleles, mating and ascus formation proceed normally and efficiently, and the spore products are fully viable (unpublished observations).

Significantly, the C3HC4 motif and two of the three membrane-spanning segments predicted for Per6p align with the C3HC4-like motifs and the two membrane-spanning segments predicted for PAF-1 and car1 (Fig. 2). The conservation of these secondary structural features indicates that these regions are important for the conformation and/or function of the proteins. Indeed, it was recently shown that the two membrane-spanning segments in Chinese hamster PAF-1 are essential (61). As proposed for car1 (2), the overall sequence similarity between Per6p and PAF-1, in conjunction with their conserved secondary structural features, suggests that Per6p may be the homolog of mammalian PAF-1.

Recently, we isolated another P. pastoris PER gene, PER4, whose predicted product also has sequence similarity to PAF-1 (21% identity, 34% similarity) (unpublished results). The Per4p sequence includes a cysteine-rich motif that is not related to the C3HC4 motif and has only 13% identity with Per6p. The existence of two different PAF-1-related PER genes in P. pastoris indicates that PAF-1-related proteins represent a family of proteins composed of at least two functionally distinct groups, with one group composed of Per4p and the other of Per6p, PAF-1, and car1.

Finally, Per6p, PAF-1, and car1 have similarity with an unpublished S. cerevisiae gene product, Crt1lp (GenBank accession no. M86538). However, the primary sequence of Crt1lp does not predict membrane-spanning segments, and its cysteine-rich motif, although akin to the C3HC4 motif, contains an alanine substituted for the histidine. Thus, it remains unclear what the relationship is between Crt1lp and the PAF-1-related proteins.

The data presented in this paper are consistent with the hypothesis that Per6p is involved in the import of peroxisomal matrix proteins. Biochemical characterization of methanol- or oleic acid-induced per6Δ cells revealed that PTS1 proteins (AOX, DHAS, CAT, and heterologously expressed luciferase) and the PTS2 protein thiolase are located in the cytosol. These biochemical data were supported by electron microscopic observations which showed that induced per6Δ cells contain only small vesicular structures that are likely to be peroxisomal remnants, as we have shown in other per mutants (37). Since peroxisome size is largely dependent on protein import, the small size of these peroxisomal remnants suggests that per6Δ is defective in peroxisomal protein matrix import and, therefore, that Per6p is required for this process. The per6-1 mutant results are also consistent with this hypothesis. In induced per6-1 cells, a minor portion of PTS1 and PTS2 proteins is imported into the peroxisomal remnants observed in these cells, although the bulk of each protein remains in the cytosol. This result suggests that the per6-1 allele is slightly leaky and allows some residual matrix protein import.

Since Per6p is a peroxisomal integral membrane protein, its role in protein import may be as part of a protein complex that functions in the translocation of proteins from the cytosol into the peroxisome matrix. As shown for other organelles like the mitochondrion (31) and the chloroplast (51), the peroxisomal protein translocation machinery is likely to be composed of

![FIG. 6. Subcellular location of selected peroxisomal proteins in wild-type and per6 mutants. Organellar pellet (P) and cytosolic supernatant (S) fractions, obtained after subcellular fractionation of wild-type (Wt), per6-1, and perΔ cells induced on methanol or oleic acid, were analyzed by immunoblots with antibodies against DHAS, AOX, thiolase, or Per6p. Each lane contains 20 μg of protein.](image-url)
many different proteins. It is tempting to speculate that the three peroxisomal cysteine-rich membrane proteins of \textit{P. pastoris} (Per6p, Per4p, and Pas7p) interact to form the core of such a protein complex.

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