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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

HANS R. WATERHAM,[1] YNE DE VRIES,[1] KIMBERLY A. RUSSELL,[1] WEIQIAO XIE,[1]†
MARTEN VEENHUIS,[2] AND JAMES M. CREGG[1]*

Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science and Technology, Portland, Oregon 97291-1000,[1] and Department of Microbiology, Biological Center, University of Groningen, 9751 NN Haren, The Netherlands[2]

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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 Per6p and PAF-1 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 (β-oxidation), purines, D-amino acids, and pipecolic 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 peroxisomal disorders, several of which are lethal (for a review, see reference 66). These disorders range in cause from 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 the peroxisomal assembly factor 1 (PAF-1), a 35-kDa peroxisomal integral membrane protein containing the cysteine-rich C3HC4 motif (54). The second 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 organellar 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_5H/QL, was initially identified at the amino terminus of 3-keto-acyl coenzyme A thiolase of mammals (46, 56) and yeasts (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

* Corresponding author. Mailing address: Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science and Technology, P.O. Box 91000, Portland, OR 97291-1000. Phone: (503) 690-1217. Fax: (503) 690-1464. Electronic mail address: cregg@admin.ogi.edu.

† Present address: Department of Genetics, Stanford University, Stanford, CA 94305.

TABLE 1. *P. pastoris* strains used

Strain	Genotype	Source or reference
JC100	Wild type	NRRL Y-11430
GS115	*his4*	6
GS200	*his4 arg4*	This study
JC114	*per6*	This study
JC116 (*per6-1*)	*per6 his4*	This study
JC214 (*per6*Δ)	*per6*::*SHIS4 his4 arg4*	This study

their inability to grow on substrates that require peroxisomes for their metabolism, whereas they are viable when grown on non-peroxisome-requiring substrates. Several genes essential for peroxisome biogenesis have been cloned by functional complementation of such mutants, providing valuable insight into the biogenesis of the organelles (e.g., 5, 13, 14, 26–28, 37, 42, 55, 58, 67). The suitability of the methylotrophic yeast *P. pastoris* as a model system is underscored by the recent identification of the human homolog of the *P. pastoris PAS8* gene, whose product is a PTS1 receptor candidate and is mutated in one complementation group of Zellweger syndrome patients (11, 69). Thus, studies of a *P. pastoris* peroxisomal biogenesis gene have resulted in the identification of a human disease gene and its etiology at the molecular level.

Previously, we and others described the isolation of a collection of peroxisome biogenesis-defective mutants of *P. pastoris* (24, 38). These mutants are defective in the ability to grow on methanol and oleic acid, substrates that require peroxisomal enzymes to be metabolized, but grow well on other non-peroxisome-requiring substrates, such as glucose, glycerol, and ethanol. Like Zellweger syndrome cells, these mutants lack normal peroxisomes and the peroxisomal matrix enzyme catalase is located in the cytosol. Here, we report the cloning of the *PER6* gene by functional complementation of a *P. pastoris per6* mutant. We show that *PER6* encodes a peroxisomal integral membrane protein that is essential for peroxisome biogenesis in *P. pastoris*. Although Per6p is a novel protein, it has significant overall amino acid sequence similarity with PAF-1, a peroxisomal integral membrane protein that is essential for human peroxisome biogenesis and is defective in one of the Zellweger syndrome complementation groups (53, 54).

MATERIALS AND METHODS

Strains, media, and microbial 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 III fermentor (New Brunswick Scientific, Edison, N.J.) in a basic mineral medium composed of 0.2 g of $MgSO_4 \cdot 7\ H_2O$ per liter, 1 g of KH_2PO_4 per liter, 0.5 g of yeast extract per liter, 1 ml of a concentrated (1,000×) trace salts solution (see below), and 40 μg of biotin per liter. $(NH_4)_2SO_4$ (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 trace salts solution was composed of 4 g of $ZnCl_2$ per liter, 1 g of $MnSO_4 \cdot H_2O$ per liter, 1.5 g of $CaCl_2$ per liter, 1 g of $FeCl_3 \cdot 6\ H_2O$ per liter, 0.05 g of H_3BO_3 per liter, 0.3 g of $CuSO_4 \cdot 5\ H_2O$ per liter, and 0.5 g of $Na_2MoO_4 \cdot 2\ H_2O$ per liter. For growth of auxotrophic strains, histidine or arginine was added to a final concentration of 50 μg/ml. The sporulation (mating) medium and procedures for classical genetic manipulation of *P. pastoris* have been described previously (38). Transformations of *P. pastoris* were performed by the spheroplast method (6) or by electrotransformation (1). Cultivation of *Escherichia coli* strains (MC1061, DH5α, and TB1) and standard recombinant DNA techniques were performed essentially as described previously (49).

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 YND plates, the transformants were collected from the plates, pooled, and inoculated in liquid YNM medium at a starting optical density at 600 nm (OD_{600}) of 0.1. After 3 days of incubation at 30°C, growth of the 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, one which, upon retransformation 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) *Cla*I 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 *Cla*I-*Dra*I subfragment was subcloned into *Cla*I- and *Hin*cII-, and *Cla*I- and *Eco*RV-digested, pBluescript II SK+ (Stratagene, La Jolla, Calif.), and a series of nested deletions was 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 certain regions. For analysis of DNA and amino acid sequences, MacVector software (IBI, New Haven, Conn.) or PCGENE release 6.8 (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 *PER6* disruption strain. To disrupt the wild-type *PER6* gene, a 4-kb *Bam*HI subfragment from pYT6 was first subcloned into the unique *Bam*HI site of pBR322, resulting in a plasmid named pRW6. Next, a 4-kb *Bam*HI-*Nru*I fragment from pYM8 (6) containing the *Saccharomyces cerevisiae HIS4* (*SHIS4*) gene was ligated into *Bam*HI- and *Sma*I-digested pBW5, a derivative of pUC19 in which a *Sac*I site had been converted into a *Mlu*I site by insertion of an oligonucleotide adapter. The *SHIS4* fragment was released from pBW5 with *Bam*HI and *Mlu*I and inserted into pRW6 digested with *Bgl*II and *Mlu*I. This resulted in a deletion of 612 bp of the *PER6* open reading frame. The resulting plasmid, named pUZ12, was digested by *Bam*HI to yield a 7.9-kb fragment composed of the *SHIS4* gene flanked by *PER6* 5′ and 3′ sequences and transformed into *P. pastoris* GS200. 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 *per6*Δ 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 *Bgl*II-*Hin*cII fragment from pYT6 in the MBP reading frame of pMAL-c2, an adapter oligonucleotide (5′-AATTCCGGATCCCTGCAGGATATCA-3′) was inserted between the *Eco*RI and *Hin*dIII site of pMAL-c2 to create an in-frame *Bam*HI site. A second adapter oligonucleotide (5′-GAGGCCTCTGCA-3′) was then inserted into the *Pst*I site of the vector to create a unique *Stu*I site. The resulting plasmid, pBW2, was digested with *Bam*HI and *Stu*I, and the *Bgl*II-*Hin*cII fragment from pYT6 was inserted to produce the MBP-Per6p expression plasmid pMW6. Expression of the MBP-Per6p hybrid protein under control of Ptac in *E. coli* TB1 was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside to exponentially growing cultures. Purification of the MBP-Per6 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 transformed 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 EGTA [ethylene glycol-bis(β-aminoethyl 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 passaged 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 per6*Δ disruption mutant JC214. Concentration and buffer exchange of the final flowthrough were performed with 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 (AOX) 1 gene promoter (*AOX1p*). The wild-type and *per6*Δ strains were transformed with the *ARG4*-based *AOX1p-LUC* plasmid pJAH23 (a gift from S. Subramani, University of California at San Diego). The *per6-1* mutant was transformed with pHWO17, a plasmid composed of the *AOX1p-LUC* expression cassette from pJAH23 subcloned as a *Bgl*II-*Pvu*II

fragment into the *Bgl*II and blunted *Cla*I sites of the *HIS4*-based vector pHIL-A1 (Invitrogen, San Diego, Calif.).

Subcellular fractionation. Wild-type and *per6* mutant cells were pregrown in YPD medium, transferred during logarithmic growth phase by centrifugation into YNM or YNO medium, and then induced for 6 h at 30°C. Subcellular fractionations of these cells were performed as described previously (38). In addition, subcellular fractionations of cells from continuous cultures were performed by the following method. After harvesting by centrifugation (5 min, 6,000 × *g*) and washing with an 0.25 volume of water, the cells were resuspended at an OD_{600} of 37.5 in 20 mM potassium phosphate buffer (KP_i, pH 7.5) supplemented with 20 mM β-mercaptoethanol. Following incubation for 15 min at 30°C, cells were harvested, washed once with KP_i alone and once with KP_i supplemented with 1.2 M sorbitol, and finally suspended at an OD_{600} of 50 in KP_i with 1.2 M sorbitol. Cells were converted to protoplasts by adding 0.8 mg of Zymolyase 100T (ICN, Costa Mesa, Calif.) per 500 OD_{600} 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 OD_{600} of 175 in MES^+ buffer (5 mM 4-morpholineethanesulfonic acid, 1 mM $MgCl_2$, 1 mM EDTA, and 0.1% ethanol) supplemented with 1 M sorbitol, 1 mM phenylmethylsulfonyl fluoride, and 0.21 mg of NaF per ml by 10 strokes in a Potter-Elvehjem tissue grinder. To remove cell debris, nonlysed protoplasts, and nuclei, the homogenate was centrifuged at 2,500 × *g* for 10 min. The resulting supernatant was saved, and the pellet was suspended in MES^+ buffer supplemented with 1 M sorbitol, phenylmethylsulfonyl fluoride, and NaF; suspension was followed by 10 more strokes and centrifugation at 2,500 × *g* for 10 min. The pooled supernatants 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 pellet, 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 OD_{600} 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.

Protein extractions with Na_2CO_3 (68), urea (7), and Triton X-114 (4) were performed as described elsewhere.

Biochemical methods. Peroxisomal AOX (65) 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 bicinchoninic 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 Electrophoretic Transfer 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, AOX, dihydroxyacetone synthase (DHAS), or thiolase (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 sequence data for *P. pastoris PER6* are available from EMBL, GenBank, and DDBJ under accession number X96945.

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 *Cla*I 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 *Cla*I 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 *Cla*I fragment revealed one long 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 negatively charged residues (67) than positively 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]). Significant similarity 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*

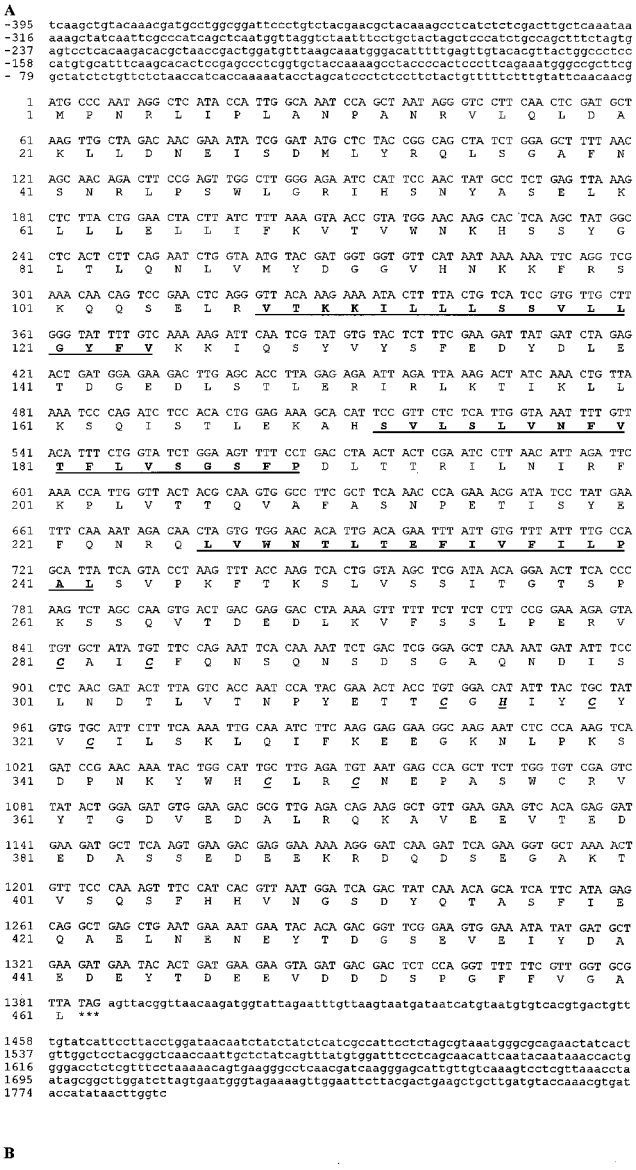

FIG. 1. *PER6* sequence and hydropathy plot. (A) Nucleotide sequence and deduced amino acid sequence of the *P. pastoris PER6* gene. Membrane-spanning segments as predicted by the method of Klein et al. (30) are in boldface and underlined. The C3HC4 motif is indicated in boldface italics and underlined. (B) Hydropathy profile of the predicted amino acid sequence of Per6p according to the method of Kyte and Doolittle (33) with a window size of 15 amino acids.

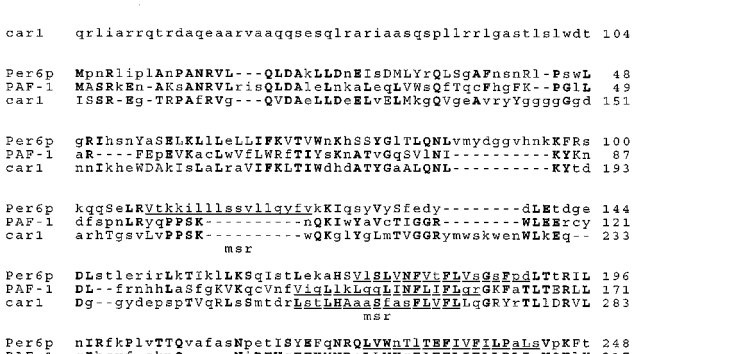

FIG. 2. Alignment of the predicted amino acid sequences of *P. pastoris* Per6p, human PAF-1, and *P. anserina* car1. Amino acid residues identical in at least two sequences are shown in boldface capitals, similar residues are shown in capitals, and nonsimilar residues are shown in lowercase. Similar residues are defined as follows: A, S, T; D, E; N, Q; R, K; I, L, M, V; F, Y, W. Membrane-spanning segments for the proteins are predicted by the method of Klein et al. (30) and underlined. The C3HC4 motif is indicated by asterisks.

(*SHIS4*) gene (Fig. 3A). The *per6*Δ::*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^+ transformant that was unable to grow on methanol indicated a correctly targeted chromosomal integration (Fig. 3B). The *per6*Δ::*SHIS4* strain JC214 (*per6*Δ) 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^-. In addition, after sporulation, approximately 500 spore products were examined, and they also were Mut^-. Together, these results demonstrate that the *per6-1* and the *per6*Δ 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 *per6*Δ 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-

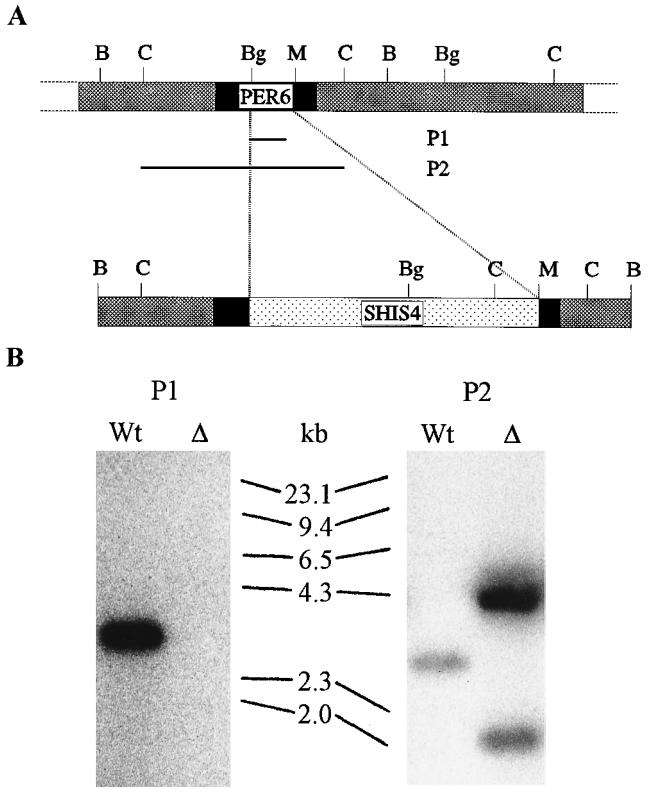

FIG. 3. Disruption of *PER6*. (A) *PER6* DNA sequences (black box) encoding Per6p amino acid residues 163 to 367 were replaced by a DNA fragment containing the *S. cerevisiae HIS4* gene (*SHIS4*; stippled box) and integrated into the *P. pastoris* genome (gray boxes) by homologous recombination. (B) Correct targeting of the *per6*Δ::*SHIS4* fragment is demonstrated by Southern blot analysis with *Cla*I-digested genomic DNA of wild type (Wt) and *per6*Δ strain JC214 (Δ). The DNA fragments used as probes (P1 and P2) are indicated in panel A.

limited continuous culture was further fractionated into peroxisomes and mitochondria by sucrose-density gradient centrifugation. Fractions were collected from the gradient and assayed for peroxisomal CAT and mitochondrial cytochrome *c* oxidase activities (Fig. 4B). Immunoblotting of fractions from this gradient revealed that Per6p had the same distribution as CAT, indicating a peroxisomal location for Per6p (Fig. 4C). Similar results were obtained with sucrose gradients of organelles isolated from methanol-grown cells (not shown). To examine the prediction that Per6p is an integral membrane protein, organelle fractions were subjected to carbonate, urea, and Triton X-114 extractions. After carbonate or urea extractions and subsequent centrifugation, Per6p was found in the membrane pellet fractions. In contrast, the peroxisomal matrix enzyme AOX was located in the supernatant (Fig. 4D). The same results were obtained when these extractions were performed with purified peroxisomes (not shown). After Triton X-114 extraction of peroxisomal membranes, Per6p was partitioned to the detergent phase (Fig. 4D). Thus, all three extraction methods indicated that Per6p is a peroxisomal integral membrane protein. The location of Per6p in the peroxisomal membrane was also studied immunocytochemically with sections of methanol-grown wild-type *P. pastoris*. In these sections, gold labeling was confined to the peroxisomal membranes, which confirmed the biochemical data that Per6p is located in the peroxisomal membrane (Fig. 5E).

***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 *per6*Δ 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 *per6*Δ 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-

TABLE 2. Distribution of peroxisomal, mitochondrial, and cytosolic marker enzyme activities in organelle pellet and cytosolic supernatant fractions obtained by subcellular fractionation of methanol- or oleic acid-induced wild-type, *per6-1*, and *per6*Δ cells[a]

C source	Strain	Enzyme[b]	P	S	P/S ratio
Methanol	WT	CAT	47.85	104.90	0.46
		AOX	0.51	0.52	0.98
		Cyt *c* oxidase	1,274.00	36.00	35.39
		GAPDH	ND	1,050.00	
	per6-1	CAT	2.53	69.09	0.04
		AOX	0.15	0.01	15.00
		Cyt *c* oxidase	719.90	96.70	7.44
		GAPDH	23.30	139.70	0.17
	*per6*Δ	CAT	2.55	56.50	0.05
		AOX	ND	ND	
		Cyt *c* oxidase	1,122.40	119.00	9.43
		GAPDH	43.30	2,825.90	0.02
Oleic acid	WT	CAT	95.63	37.94	2.52
		Luciferase	35.97	14.51	2.48
		Cyt *c* oxidase	629.00	8.00	78.63
		GAPDH	56.94	5,612.00	0.01
	per6-1	CAT	2.76	94.46	0.03
		Luciferase	19.15	306.97	0.06
		Cyt *c* oxidase	574.00	9.00	63.77
		GAPDH	87.60	4,944.00	0.02
	*per6*Δ	CAT	3.65	79.00	0.05
		Luciferase	2.99	152.06	0.02
		Cyt *c* oxidase	158.00	4.00	39.50
		GAPDH	269.29	1,591.00	0.17

[a] Abbreviations: P, pellet; S, supernatant; Cyt *c*, cytochrome *c*; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; WT, wild type; ND, not detected.
[b] Activity units: CAT, ΔE_{240} per minute per milligram; AOX, micromoles of product per minute per milligram; cytochrome *c* oxidase and glyceraldehyde 3-phosphate dehydrogenase, nanomoles of product per minute per milligram; luciferase, arbitrary light units per milligram of protein.

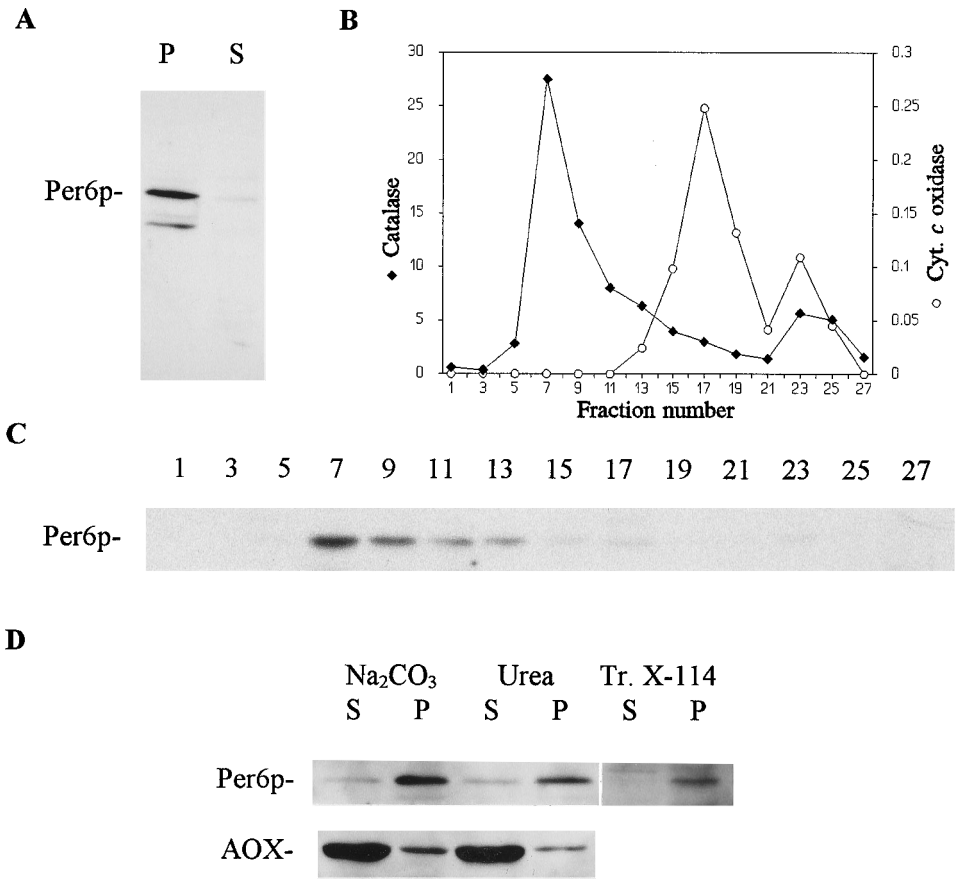

FIG. 4. Per6p is a peroxisomal integral membrane protein. (A) Organelle pellet (P) and cytoplasmic supernatant (S) fractions obtained after subcellular fractionation of oleic acid-grown wild-type cells were analyzed by immunoblot with affinity-purified anti-Per6p antibodies. Each lane contains 20 μg of protein. (B) Sucrose density gradient profile of the organellar pellet obtained from oleic acid-grown wild-type cells. Fractions collected from the gradient were assayed for peroxisomal CAT and mitochondrial cytochrome *c* oxidase activity. (C) Equal volumes of every odd-numbered fraction of the sucrose density gradient in panel B were analyzed by immunoblot with the anti-Per6p antibodies. (D) A total of 25 μg of the organelle pellet of methanol-grown wild-type cells was extracted with 0.1 M sodium carbonate (pH 11) or 8 M urea. In addition, peroxisomal membranes were extracted with Triton X-114. After extraction, samples were centrifuged, and supernatant and pellet fractions were analyzed by immunoblot with anti-Per6p and anti-AOX antibodies.

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 *per6*Δ 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 *per6*Δ 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-

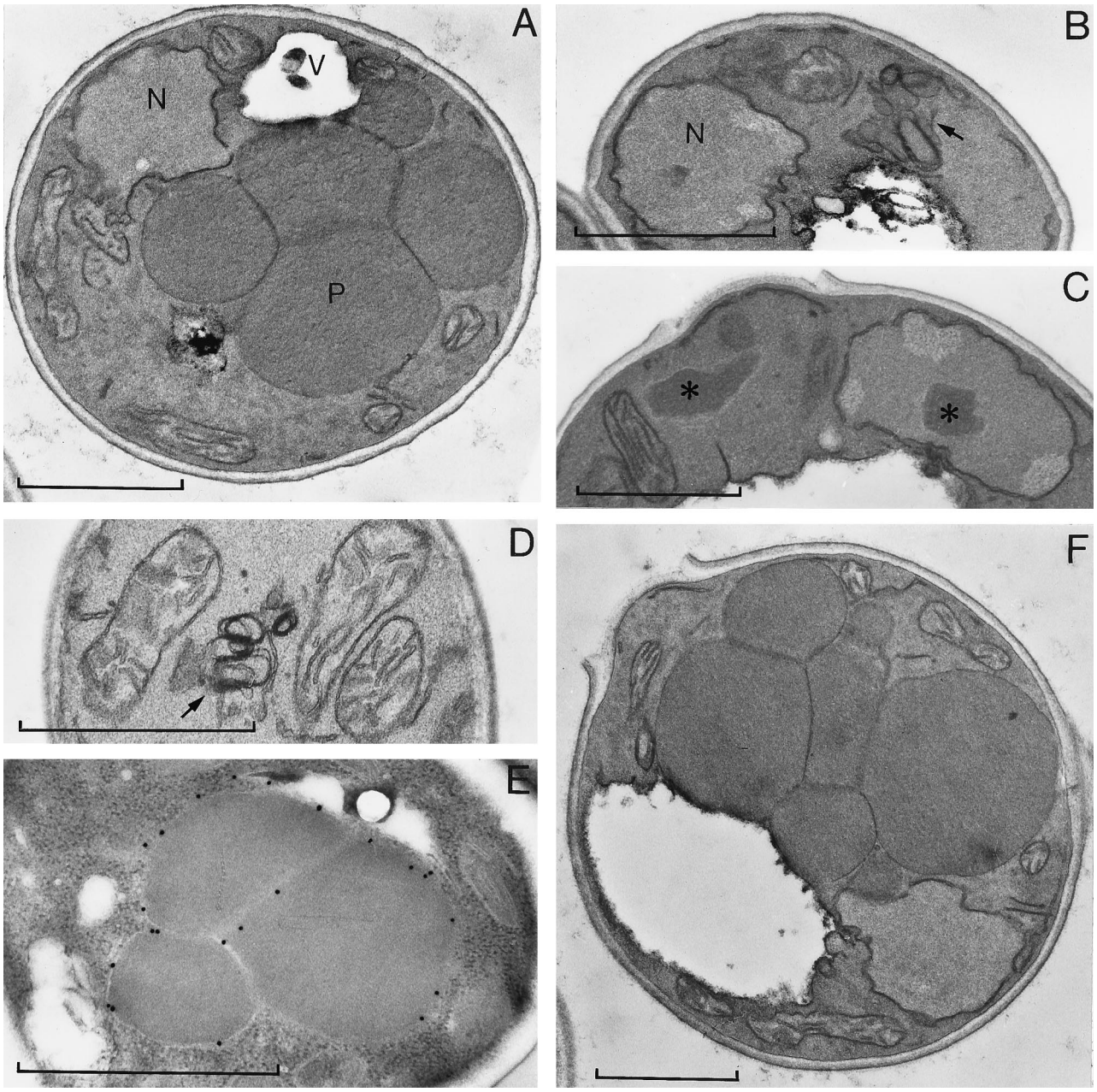

FIG. 5. Electron micrographs showing subcellular morphology of selected *P. pastoris* strains. (A) Proliferation of peroxisomes in methanol-grown wild-type cells. (B and D) In methanol-induced cells of *per6-1* (B) as well as *per6*Δ (D), vesicular structures (arrows) that are likely to be peroxisomal remnants are induced. (C) These cells also frequently contain electron-dense protein aggregates (asterisks). (E) Immunocytochemistry using the specific antibodies against Per6p revealed a location of Per6p on the peroxisomal membrane. (F) The *per6-1* mutant is complemented by pYT6, resulting in methanol growth and the appearance of normal peroxisomes. (A to D and F) $KMnO_4$ fixation; (E) aldehyde-unicryl-uranyl acetate. Abbreviations: N, nucleus; P, peroxisome; V, vacuole. Bars, 0.5 μm.

mal remnants. In contrast, the small amounts of thiolase and luciferase in the organelle pellet of the *per6*Δ 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

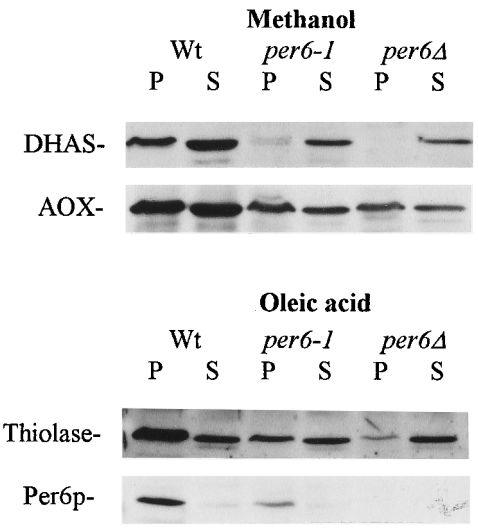

FIG. 6. Subcellular location of selected peroxisomal proteins in wild-type and *per6* mutants. Organelle pellet (P) and cytosolic supernatant (S) fractions, obtained after subcellular fractionation of wild-type (Wt), *per6-1*, and *per6*Δ cells induced on methanol or oleic acid, were analyzed by immunoblots with antibodies against DHAS, AOX, thiolase, or Per6p. Each lane contains 20 μg of protein.

receptor (42, 59); Pas4p, a ubiquitin-conjugating protein (5); and Per3p, a protein involved in protein import (37).

The calculated molecular mass of Per6p is 52 kDa. However, on immunoblots, antibodies against Per6p recognized a major protein species at 58 kDa. The discrepancy between the calculated and empirical sizes of Per6p can be explained by the physical properties of the protein, such as its overall negative charge, which results in a low pI (4.6), which may retard migration in SDS-polyacrylamide gels.

The carboxy-terminal half of Per6p contains a C3HC4 motif (or RING motif), a cysteine-rich region which defines a family of proteins involved in a variety of physiological functions (17, 32). Since several proteins of this family are located in the nucleus, it is assumed that the motif is involved in DNA binding. However, other proteins, including Per6p, are not located in the nucleus, and therefore, it has been suggested that the motif may participate in other processes such as protein-protein interaction (32). Although the precise role of the C3HC4 motif remains unclear, its importance in peroxisome biogenesis is suggested by its presence in several peroxisomal integral membrane proteins required for peroxisome biogenesis. In addition to Per6p, the motif is found in Per8p of *H. polymorpha* (58) and its *P. pastoris* homolog Pas7p (28) and in the mammalian PAF-1 proteins (54, 60, 61).

Per6p contains significant overall amino acid sequence similarity with the mammalian PAF-1 proteins and *P. anserina* car1. The mammalian PAF-1 proteins are 35-kDa peroxisomal integral membrane proteins that are essential for peroxisome biogenesis. Defects in human PAF-1 result in a lethal disorder called Zellweger syndrome. In cells from these patients, normal peroxisomes are absent and peroxisomal matrix proteins are located in the cytosol (44, 54), the same phenotype we observe in our *per6* mutants. The amino acid sequence of *P. pastoris* Per6p has 29% identity with that of human PAF-1. This level of identity is comparable to that shared by other yeast and human homologs, including peroxisomal proteins such as the putative *P. pastoris* and human PTS1 receptors Pas8p and Pxr1p/PTS1R, which are 30% identical (11, 16, 42, 69), and the *S. cerevisiae* and human ATP-binding cassette 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 *per6*Δ 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, Crt1p (GenBank accession no. M86538). However, the primary sequence of Crt1p 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 Crt1p 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 *per6*Δ 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 *per6*Δ 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 *per6*Δ 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

many different proteins. It is tempting to speculate that the three peroxisomal cysteine-rich membrane proteins of *P. pastoris* (Per6p, Per4p, and Pas7p) interact to form the core of such a protein complex.

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