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Isolation and Characterization of Pas2p, a Peroxisomal Membrane Protein Essential for Peroxisome Biogenesis in the Methylotrophic Yeast *Pichia pastoris**

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The *pas2* mutant of the methylotrophic yeast *Pichia pastoris* is characterized by a deficiency in peroxisome biogenesis. We have cloned the *PpPAS2* gene by functional complementation and show that it encodes a protein of 455 amino acids with a molecular mass of 52 kDa. In a *Pppas2* null mutant, import of both peroxisomal targeting signal 1 (PTS1)- and PTS2-containing proteins is impaired as shown by biochemical fractionation and fluorescence microscopy. No morphologically distinguishable peroxisomal structures could be detected by electron microscopy in *Pppas2* null cells induced on methanol and oleate, suggesting that PpPas2p is involved in the early stages of peroxisome biogenesis. PpPas2p is a peroxisomal membrane protein (PMP) and is resistant to extraction by 1 M NaCl or alkaline sodium carbonate, suggesting that it is a peroxisomal integral membrane protein. Two hydrophobic domains can be distinguished which may be involved in anchoring PpPas2p to the peroxisomal membrane. PpPas2p is homologous to the *Saccharomyces cerevisiae* Pas3p. The first 40 amino acids of PpPas2p, devoid of the hydrophobic domains, are sufficient to target a soluble fluorescent reporter protein to the peroxisomal membrane, with which it associates tightly. A comparison with the membrane peroxisomal targeting signal of PMP47 of *Candida boidinii* revealed a stretch of positively charged amino acids common to both sequences. The role of peroxisomal membrane targeting signals and transmembrane domains in anchoring PMPs to the peroxisomal membrane is discussed.

Peroxisomes (microbodies) comprise an inducible and versatile eukaryotic subcellular compartment that plays a key role in a number of metabolic pathways. A general feature is its involvement in H_2O_2 metabolism and the β-oxidation of fatty acids (Van den Bosch *et al.*, 1992; Wiemer and Subramani, 1994). The importance of peroxisomes is emphasized by the existence of severely debilitating, and often lethal, human diseases in which peroxisome biogenesis is impaired (Lazarow and Moser, 1989). In fungi, peroxisomes are the sole site for β-oxidation of fatty acids and, in methylotrophic yeasts, are essential for the oxidation of methanol (Kunau *et al.*, 1987; Veenhuis, 1992). Much progress has been made in recent years delineating the molecular requirements for import of peroxisomal matrix proteins (metabolic enzymes). Typically, these proteins are synthesized on cytosolic polysomes and imported posttranslationally without further modifications. Two evolutionary conserved peroxisomal targeting signals (PTSs)[1] for matrix proteins have been identified (Subramani, 1993). Most frequently encountered is PTS1, consisting of a COOH-terminal tripeptide with the consensus sequence S/A/C-K/R/H/-L/M (Gould *et al.*, 1989; Keller *et al.*, 1991). PTS2 is found at the amino termini of a smaller subset of peroxisomal proteins and conforms to the consensus RL-X_5-H/QL (Swinkels *et al.*, 1991; Osumi *et al.*, 1991; Erdmann, 1994; Glover *et al.*, 1994; Faber *et al.*, 1995). Separate import pathways exist for PTS1- and PTS2-containing proteins since both in yeast and mammalian cells import defects have been observed excluding either PTS1- or PTS2-containing proteins from the peroxisome (McCollum *et al.*, 1993; Motley *et al.*, 1994; Marzioch *et al.*, 1994; Slawecki *et al.*, 1995; Zhang and Lazarow, 1995). Functional complementation of the yeasts has revealed the PTS1 (PpPas8p, ScPas10p, HpPer3p, and YlPay32p) and PTS2 receptors (ScPas7p or ScPeb1p) that specifically interact with their cognate signals (McCollum *et al.*, 1993; Van der Leij *et al.*, 1993; Marzioch *et al.*, 1994; Zhang and Lazarow, 1995, 1996; Terlecky *et al.*, 1995).

Besides the yeast mutants with a selective import defect for matrix proteins, a large collection of strains has been described which show a general defect in peroxisome assembly (*pas*, *per pay*, or *peb* mutants; Erdmann *et al.*, 1989; Cregg *et al.*, 1990; Gould *et al.*, 1992; Liu *et al.*, 1992; Van der Leij *et al.*, 1992; Elgersma *et al.*, 1993; Nuttley *et al.*, 1993; Zhang and Lazarow, 1993). In these strains the bulk of the peroxisomal matrix proteins is found in the cytosol. Some contain peroxisomes with an aberrant morphology reminiscent of the peroxisomal ghost structures found in fibroblast cell lines derived from patients with peroxisomal disorders (Wiemer *et al.*, 1989).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Z72390.

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[1] The abbreviations used are: PTS(s), peroxisomal targeting signal(s); PMP, peroxisomal membrane protein; mPTS, peroxisomal membrane targeting signal; PCR, polymerase chain reaction; kb, kilobase; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; TMD, transmembrane domain; PAS, peroxisome assembly.

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TABLE I
P. pastoris strains

Strain	Genotype (expression construct)	Source
PPY1	21-1 Wild type	Y-11430, NRRL
PPY4	*Pphis4*	Gould *et al.* (1992)
PPY12	*Pphis4, Pparg4*	Gould *et al.* (1992)
PPY21	*Pppas2, Pparg4*	Gould *et al.* (1992)
SEW1 (*Pppas2* null)	*Pphis4, Pppas2*::*PpARG4*	This paper
SEW2	*Pppas2*::*PpARG4, Pphis4*::pTW51 (P_{AOXI}GFP-PTS1)	This paper
SEW3	*Pppas2*::*PpARG4, Pphis4*::pTW66 (P_{GAPDH}PTS2-GFP)	This paper
STW1	*Pparg4, Pphis4*::*p*TW51 (P_{AOXI}GFP-PTS1)	This paper
STW2	*Pparg4, Pphis4*::pTW66 (P_{GAPDH}PTS2-GFP)	This paper
SKF1	*Pparg4, Pphis4*::pKNSD77 (P_{AOXI}1-40-GFP)	This paper

Complementing genes have been isolated and characterized for most of these mutants. Several of the encoded proteins were shown to be membrane-associated. The functions and molecular requirements for targeting of these peroxisomal membrane proteins (PMPs), which do not contain a PTS1 or PTS2, are largely unknown.

Here we report the cloning of the *Pichia pastoris PAS2* gene, encoding a peroxisomal membrane protein (PpPas2p) which is essential for peroxisome biogenesis. The phenotype of the *Pppas2* null strain is examined, and the peroxisomal membrane-targeting signal (mPTS) of PpPas2p is delineated.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions—The *P. pastoris* strains used in this study are listed in Table I. Yeast strains were grown at 30 °C in YPD (1% w/v yeast extract, 2% w/v Bacto-peptone, 2% w/v dextrose), YPM (1% w/v yeast extract, 2% w/v Bacto-peptone, 0.5% v/v methanol), YPOT (1% w/v yeast extract, 2% w/v Bacto-peptone, 0.2% v/v oleate, 0.02% v/v Tween 40), or in synthetic medium consisting of 0.67% w/v yeast nitrogen base, supplemented with 50 μg/ml of the appropriate amino acids and with one of the following carbon sources: 2% w/v dextrose (SD), 0.5% v/v methanol (SM), or 0.2% v/v oleate and 0.02% v/v Tween 40 (SOT). Bacto-agar (2% w/v) was added for solid media. Mating, sporulation, and random spore analysis were performed as described by Gould *et al.* (1992).

Molecular Biological Techniques—Strain DH5α was used in all cloning procedures involving plasmid propagation. Enzyme digests, cloning techniques, plasmid isolations, polymerase chain reactions (PCRs) and Southern blotting were performed according to standard protocols. DNA sequencing was performed according to Sanger *et al.* (1977), using the Sequenase kit (U. S. Biochemical Corp.).

P. pastoris strains were transformed by electroporation according to Rickey (1990). DNA was isolated from yeast as described by Gould *et al.* (1992).

Cloning and Sequencing of the PpPAS2 Gene—Strain PPY21 was transformed with a *P. pastoris* genomic library described by Gould *et al.* (1992). The *PpPAS2* gene was identified by functional complementation of the *Pppas2* mutant, selecting for restoration of growth on SM and SOT media. Physical maps of the inserts from complementing plasmids were determined by restriction analysis. Fragments were subcloned into plasmid pSG560 (Gould *et al.*, 1992) and reintroduced into strain PPY21. A 1.8-kb genomic DNA fragment with the ability to complement PPY21 for growth on methanol and oleate was cloned in both orientations into pBSII KS (Stratagene), yielding pBS-PAS2A and pBS-PAS2B. A set of plasmids with nested deletions generated by exonuclease III and S1 nuclease (Erase-a-Base kit, Promega, Madison, WI) was used for sequencing both DNA strands.

Construction of the Pppas2 Null Mutant—An *Eco*RI-*Acc*I fragment of 1,230 base pairs (nucleotides 116-1346), encompassing most of the *PpPAS2* coding region, was replaced by a 2.0-kb *Eco*RI-*Hin*dIII fragment containing the *P. pastoris ARG4* gene (see Fig. 2). The *Acc*I and *Hin*dIII sites were filled in using Klenow polymerase. Strain PPY12 was transformed with a linear DNA fragment containing the *PpARG4* gene and *PpPAS2* flanking regions. Transformants were selected for arginine prototrophy on SD plates and tested for growth on SM and SOT media. Cells that failed to utilize both carbon sources were analyzed by diagnostic PCR and Southern blotting (Southern, 1975) to confirm integration of the disruption construct at the correct chromosomal locus. This strain was called SEW1 (*Pppas2* null).

Plasmids—Plasmid pJAH35-PAS2 was obtained by cloning the 1.8-kb genomic DNA fragment containing the *PpPAS2* gene as a *Sma*I fragment (polylinker sites) into the *Eco*RI site (blunted with Klenow polymerase) of pJAH35, a pBR322-based vector containing the *PpHIS4* gene and a *P. pastoris* autonomously replicating sequence (*PARS2*, Cregg *et al.*, 1985).

Plasmid pTW51, containing the green fluorescent protein-PTS1 (GFP-PTS1) construct under control of the methanol-inducible alcohol oxidase promoter (P_{AOXI}), was obtained by cloning an *Eco*RI PCR product of the GFPS65T mutant allele (Heim *et al.*, 1995) in the *P. pastoris* integrating vector pHIL-D2 (Invitrogen, San Diego). The primer at the 3′ end contained the appropriate codons to include SKL (PTS1 signal) in the predicted translation product (Monosov *et al.*, 1996). Plasmid pTW66, containing the PTS2-GFP construct under control of the constitutive glyceraldehyde 3-phosphate dehydrogenase promoter (P_{GAPDH}; gift of Dr. J. M. Cregg, Oregon Graduate Institute of Science and Technology, Portland, OR) was constructed by cloning the GFPS65T allele in-frame with a part of the *ScFOX3* sequence (Glover *et al.*, 1994) encoding the NH_2-terminal 16 amino acids of the *Saccharomyces cerevisiae* 3-oxoacyl-CoA thiolase in vector pHIL-D2.

A hybrid gene encoding the NH_2-terminal 40 amino acids of PpPas2p and GFP (1–40-GFP) was constructed as follows. By PCR, an Asp-718 and a *Bgl*II site were introduced upstream of the *PpPAS2* open reading frame present in pBSIIKS, resulting in pKNSD44. A second PCR was performed to amplify a DNA fragment encoding the GFPS65T allele flanked by an *Eco*RI (5′) and a *Spe*I (3′) site which was inserted into *Eco*RI and *Spe*I-digested pKNSD44 resulting in pKNSD73. Finally, the hybrid gene was inserted as a 0.9-kb *Bgl*II-*Not*I DNA fragment into the polylinker of pPIC3K (Invitrogen), resulting in pKNSD77. All of the GFP expression vectors were linearized by *Sal*I digestion to target integration into the genomic *PpHIS4* locus.

Biochemical Assays, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and Western Blotting—Catalase and cytochrome *c* oxidase activities were determined according to Baudhuin *et al.* (1964) and Madden and Storrie (1987), respectively. Protein concentrations were determined according to Smith *et al.* (1985) or Bradford (1976).

SDS-PAGE and Western blotting were performed as described by Laemmli (1970) and Towbin *et al.* (1979), respectively. Blots were incubated with primary antibodies, immune complexes were visualized by the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium color reaction after incubation of blots with a goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (dilution 1:5,000, Bio-Rad). Alternatively, immune complexes were detected by the ECL technique (Amersham Corp.) after incubation of blots with a protein A-horseradish peroxidase conjugate (dilution 1:3,000, Bio-Rad).

Antibodies—An *Ase*I (filled in with Klenow polymerase)-*Hin*dIII fragment of 1,717 base pairs (amino acids 8–455) of pBS-PAS2A was cloned between the *Xba*I (filled in with Klenow polymerase) and *Hin*dIII sites of the pGEX-KG polylinker (Guan and Dixon, 1991). The resulting glutathione *S*-transferase PpPas2p fusion (GST-PpPas2p) protein was synthesized in *E. coli* DH5α as described by Guan and Dixon (1991), except that no benzamidine was used, and the cells were lysed by sonication. The largely insoluble GST-PpPas2p fusion protein was isolated by SDS-PAGE and cleaved by thrombin. The 52-kDa PpPas2p fragment was gel purified and used to immunize a rabbit (Harlow and Lane, 1988).

Anti-PpPer6p antibodies were a gift from Dr. J. M. Cregg (Oregon Graduate Institute of Science and Technology, Portland, OR), and anti-ScFox3p (anti-*S. cerevisiae* 3-ketoacyl-CoA thiolase) antibodies were a gift from Dr. W.-H. Kunau (Ruhr University, Bochum, Germany). Anti-PpPas8p, anti-PpAox1p (anti-*P. pastoris* alcohol oxidase), and anti-GFP were generated as described by McCollum *et al.* (1993) and Monosov *et al.* (1996), respectively.

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-90                GATCCATCTTTACTTTGATCATCATTGCAAAGCCATAA

-52  ACTCTGAGAGGAACACAAGTGCATACAATCACTAGATATTTGCAAAGTCG
                          AseI
 -2  TAATGTTGGAGTACACGGCAGGATTAATAAGACGAAACAAGAAAAAGTTT
       M  L  E  Y  T  A  G  L  I  R  R  N  K  K  K  F>   16

 49  CTTATATCCTCTGGAATCATAGGAGTAGGTTACTATGTAACCAAAACTAT
       L  I  S  S  G  I  I  G  V  G  Y  Y  V  T  K  T  I>   33
                   EcoRI
 99  CAATAACAAGATCCAAGAATTCCAAAACCGAATTAGAGAGGAAAACTTTG
       N  N  K  I  Q  E  F  Q  N  R  I  R  E  E  N  F>   49

149  CCAAAGAGCAAATCAAGCGTCGGTTTCACCAGACGCAAAGTGATTGCTAC
       A  K  E  Q  I  K  R  R  F  H  Q  T  Q  S  D  C  Y>   66

199  ATGACCTTTCTATCACTGCTGCCAGTACTTTGTGAACCAATTATGGACGA
       M  T  F  L  S  L  L  P  V  L  C  E  P  I  M  D  D>   83

249  TTTGCCAGTAGAGACTATCACCAAGCAGCTCCAAATTAGACGGCTAGAAA
       L  P  V  E  T  I  T  K  Q  L  Q  I  R  R  L  E>   99
                            EcoRI  BamHI
299  AACAAATTGGCAACAAAGATGTTAAGAATTCCGGATCCACTGTGCTTAGT
       K  Q  I  G  N  K  D  V  K  N  S  G  S  T  V  L  S>  116

349  GACGATTTCTCAACATCTCAAGAGGGAGCGATATCGGAGGATACTAACAA
       D  D  F  S  T  S  Q  E  G  A  I  S  E  D  T  N  K>  133

399  ACCTCCTGAACTGAAAAGTAAAAATCAATTGTGGCAAGAGCTAAAAATCA
       P  P  E  L  K  S  K  N  Q  L  W  Q  E  L  K  I>   149

449  AAGCAATAACAAGATTTCTCACGCTGATATATTGTGAATCCTTGCTGATT
       K  A  I  T  R  F  L  T  L  I  Y  C  E  S  L  L  I>   166

499  GTATTCTTACACCTTCAGCTTAATATTCTATCGCGCAAGTCTTATCTTGA
       V  F  L  H  L  Q  L  N  I  L  S  R  K  S  Y  L  E>   183

549  GACTGCAATCAGGCTGGCTTCTGAGACCCAGGGGATAGATTTGGTAGATC
       T  A  I  R  L  A  S  E  T  Q  G  I  D  L  V  D>   199

599  AAGAGTCTAATGGTGACTTTTCTGGCAACACTCAGGATGAAAACTTATCC
       Q  E  S  N  G  D  F  S  G  N  T  Q  D  E  N  L  S>   216

649  GAACAAGCATTCTTGAGTTTTAGTTGGTGGCTGCTCAACAAGGGATGGCT
       E  Q  A  F  L  S  F  S  W  W  L  L  N  K  G  W  L>   233

699  TGAGATAAAGAACAAGATTGAGCCATGTGTTGAACAACACTTTGGAGGTA
       E  I  K  N  K  I  E  P  C  V  E  Q  H  F  G  G>   249

749  TAAATCCCAGACAGCAATTGAAGATAAATGAGTTTGCCGAGCTTTTAAAC
       I  N  P  R  Q  Q  L  K  I  N  E  F  A  E  L  L  N>   266

799  AAATGTCAAAATTGTATTGACCTGAAGGTTTTAAATCTTACCGAAGAGGA
       K  C  Q  N  C  I  D  L  K  V  L  N  L  T  E  E  D>   283

849  TATACATTTGGGAGTCGGTGTTATCGAAGACCAGTCCCAGCCTGTTGGAA
       I  H  L  G  V  G  V  I  E  D  Q  S  Q  P  V  G>   299
```

FIG. 1. **Nucleotide sequence of the *PpPAS2* gene and the deduced amino acid sequence.** Shown is the translation of the *PpPAS2* sequence from the ATG, at nucleotide +1, to the termination codon (STOP), at nucleotides 1366–1368. The amino acids are in the one-letter code. Relevant restriction sites are indicated.

Preparation of Crude Yeast Lysates—Yeast cells were harvested by centrifugation, resuspended in 3 volumes (compared with the volume of the pellet) of disruption buffer (20 mM Tris-Cl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5% v/v glycerol, 1 mM dithiothreitol, 0.3 M ammonium sulfate, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) and mixed with 4 volumes of acid-washed beads. Cell suspensions were vortexed for 1 min after which the tubes were placed on ice for 2 min. This treatment was repeated five times after which the supernatants were removed. The beads were washed once with disruption buffer, and supernatants, representing the crude cell extracts, were pooled and stored at −20 °C.

Subcellular Fractionations—Strains PPY4 (wild type for *PpPAS2*) and SEW1 (*Pppas2* null mutant) were used for cell fractionation experiments. Cells were cultured in 500 ml of YPD medium to near saturation, pelleted, resuspended in 2 liters of SM medium, and incubated for 24 h. Preparation of spheroplasts and cell homogenates, as well as the subsequent differential and density gradient centrifugation, were performed according to Monosov *et al.* (1996).

Enriched organelle fractions (27,000 × *g* pellet) of strains STW1 and SKF1, grown on SM medium, were diluted 10-fold either in 10 mM Tris-Cl, pH 8.5, or 1 M NaCl in 10 mM Tris-Cl, pH 8.5, or 0.1 M Na_2CO_3, pH 11.5, and incubated on ice for 1 h. Insoluble materials were pelleted by centrifugation for 1 h at 100,000 × *g* at 4 °C in a Beckman SW 50.1 rotor. Pellet fractions were resuspended in the appropriate buffer; after centrifugation as above, the corresponding supernatants were pooled. Distributions of PpPas2p, PpPas8p, PpPer6p, catalase, and GFP fusions in all peroxisomal subfractions were analyzed by SDS-PAGE and immunoblotting.

```
899  GAAAAAGCACTAACTTTATCACAAACGCTTTATTACCACCCAAAGAGTTT
       R  K  S  T  N  F  I  T  N  A  L  L  P  P  K  E  F>   316

949  GAGTTTTTCCTTTTGCAACAGACAAATGATTTAGACTTTTTATCCCGCTT
       E  F  F  L  L  Q  Q  T  N  D  L  D  F  L  S  R  F>   333

999  CAACAATAATATCGTCAATACTGAGAGCTTGAACATGTTATTGGATGAAT
       N  N  N  I  V  N  T  E  S  L  N  M  L  L  D  E>   349

1049 TAAACAACTATCTGAACAATGCGGACATCAATCTGATAGTCAACAAATTG
       L  N  N  Y  L  N  N  A  D  I  N  L  I  V  N  K  L>   366

1099 GCTACCCTAGGTATTACCAAAGTTCTGGATGAAATAGTTCTCAACCTATT
       A  T  L  G  I  T  K  V  L  D  E  I  V  L  N  L  L>   383

1149 ACAGAAGAATCGACCGAACCCAATTTCAGATATGAATATACGTGATATTG
       Q  K  N  R  P  N  P  I  S  D  M  N  I  R  D  I>   399

1199 ATCTCAATGATTTTCCCCCCTACAAATTAGCAGCTTTGCTGGCAAACATA
       D  L  N  D  F  P  P  Y  K  L  A  A  L  L  A  N  I>   416

1249 ACCAAACAGTCTATTTCGTTGACTAACAACTCTGTGGAGAACCCTATTTT
       T  K  Q  S  I  S  L  T  N  N  S  V  E  N  P  I  L>   433
                                                   AccI
1299 AGCAGACTTAAACAATTTACCGGAACTTAATGATCTCAGCGCAAGTGTAT
       A  D  L  N  N  L  P  E  L  N  D  L  S  A  S  V>   449

1349 ACTCTAATTTTGATCCTTAGAACTCGTGAGAAATATATCAATTTCACTAC
       Y  S  N  F  D  P  STOP                              455

1399 TAAATTAACTACAGAGAATCAGACTGTTGGTTATACTGACATGAAAGGAT

1449 ACTTAGTTTTCAATTACCTAGAGTGGACCCTCATGGTATCTGCCATTGCT

1499 GCAATCCTCCAACAGCGGCTCAATTTGTGATGGCCAATGGCTACTCTTGT

1549 AATGTTGACCGCATCCATCACCGCGGCACTTTCCTAGTTTCTCAGCAAAG

1599 TGCGACGAACAGATACAGTCAATTGACTTCTTCTTGCTCTTTTTACAAGC

1649 ATTAGCAAATATTTTGGTCTCAGTGCACTGTTTGTCGCACCATTTGGCTG
                    PstI
1699 GCCATTTATCTTCTTTTTCTGCAG
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FIG. 1—*continued*

Microscopy and Immunocytochemistry—GFP fluorescence in living cells was analyzed using the fluorescein isothiocyanate channel of a Photomicroscope III fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a Planopochromat 63/1.4 (oil) objective.

Cells for electron microscopy were fixed with 1.5% w/v $KMnO_4$ for 20 min at room temperature, washed extensively with water, resuspended in 0.5% w/v uranyl acetate, and incubated for 15 min at room temperature. Samples were embedded in Epon 812 after dehydration in a graded ethanol series. Ultrathin sections were examined in a Philips 300 electron microscope.

Cells for immunocytochemistry were fixed in 3% v/v glutaraldehyde, 0.1 M sodium cacodylate, pH 7.2 for 30 min at 0 °C, dehydrated in a graded ethanol series, and embedded in Lowicryl K4 M. Immunolabeling was performed on ultrathin sections by the protein A-gold method (Slot and Geuze, 1984), using anti-ScFox3p and anti-PpPas2p as primary antibodies and goat anti-rabbit immunoglobulin conjugated to colloidal gold as secondary antibodies. Sections were poststained in 0.5% w/v uranyl acetate and examined in a Philips 300 or CM10 electron microscope.

RESULTS

Cloning of PpPAS2 by Functional Complementation—Three *Pppas2* mutants were isolated, selecting for cells deficient in peroxisome assembly (Gould *et al.*, 1992), which were unable to grow on media containing oleic acid or methanol as sole carbon sources. However, their growth was normal on glucose and nonfermentable carbon sources such as glycerol, malate, ethanol, and lactate. Through screening of a genomic DNA library, a 1.8-kb DNA fragment was obtained, which restored the ability of the mutant strain to utilize methanol and oleate. Sequencing of this DNA fragment revealed a 1,365-base pair open reading frame encoding a 455-amino acid protein (calculated molecular mass of 51,969 Da) with an estimated pI of 4.8 (Fig. 1).

Deletion of PpPAS2 Abolishes Import of PTS1- and PTS2-containing Reporter Proteins—The *PpPAS2* gene was replaced

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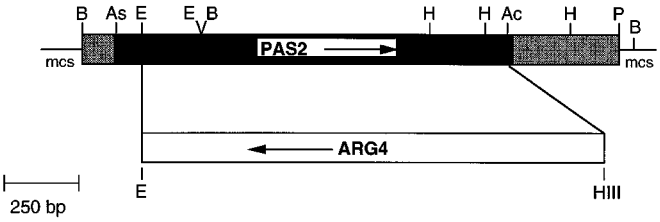

FIG. 2. **Schematic representation of the *PpPAS2* knockout construct.** The *gray bar* represents the genomic fragment cloned by functional complementation of the *Pppas2* mutant. The *black bar* marks the location of the *PpPAS2* open reading frame. The *PpPAS2* fragment that is replaced with the *PpARG4* gene is indicated. *Arrows* indicate the direction of transcription. *Ac*, *Acc*I; *As*, *Ase*I; *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dII; *HIII*, *Hin*dIII; *P*, *Pst*I; *mcs*, multiple cloning site of pBSII KS; *bp*, base pairs.

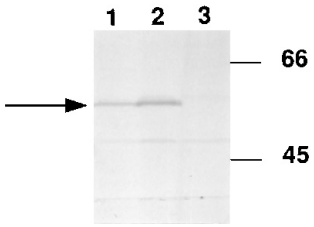

FIG. 3. **Synthesis of PpPas2p in wild type and the null mutant.** Crude extracts (100 μg of total protein) from wild type PPY1 cultured in YPD (*lane 1*), YPM (*lane 2*), and the null mutant induced in YPM (*lane 3*) were subjected to SDS-PAGE. The proteins were transferred to nitrocellulose and the blot incubated with anti-PpPas2p (dilution 1:5,000). Molecular mass markers are indicated on the *right*. The *arrow* marks PpPas2p, migrating at 52 kDa.

with the *PpARG4* gene (Fig. 2). The resulting *Pppas2* null mutant showed normal growth on glucose but was unable to metabolize methanol and oleate, as observed for the original mutants. In a genetic cross between the *PpPas2* null mutant and the *PpPas2* mutant, no complementation was observed for growth on methanol or oleate. This indicates that the identified open reading frame was not that of a suppressor gene. Reintroduction of *PpPAS2* into the null mutant restored the ability to grow on methanol and oleate (results not shown). Notably, the cloned 5′-noncoding region of only 90 nucleotides was sufficient to direct expression of the *PpPAS2* gene on both oleate and methanol (see below).

Antibodies raised against PpPas2p specifically detected a protein of approximately 52 kDa in lysates of wild type *P. pastoris* cells grown on YPD (Fig. 3, *lane 1*), and only a moderate 3–5-fold induction of the PpPas2p was noted on methanol (Fig. 3, *lane 2*) and oleate (results not shown). As expected, no PpPas2p was detected in lysates from the null mutant (Fig. 3, *lane 3*).

The import of peroxisomal matrix proteins was assessed by determining the intracellular location of GFP fused either to a PTS1 or PTS2 sequence. A GFP-PTS1 fusion protein was expressed in strains STW1 (producing PpPas2p and GFP-PTS1) and SEW2 (producing GFP-PTS1 but lacking PpPas2p). Upon induction on methanol, the GFP-PTS1 protein was directed to the peroxisomes of STW1 cells as judged by the intense fluorescent spots representing the large clustered peroxisomes (Fig. 4*A*). These results are in agreement with data published by Monosov *et al.* (1996). Likewise, a hybrid protein consisting of the NH_2-terminal 16 amino acids of *S. cerevisiae* thiolase and GFP (PTS2-GFP) was targeted to the peroxisomes in oleate-grown STW2 (expressing PpPas2p and PTS2-GFP) (Fig. 4*B*). This implies that the sequence identified as the PTS for thiolase is also properly recognized as such in *P. pastoris*. In strains SEW2 and SEW3, lacking PpPas2p, both GFP-PTS1 (Fig. 4*C*) as well as PTS2-GFP (Fig. 4*D*) were present in the cytoplasm as indicated by the diffuse fluorescent signal.

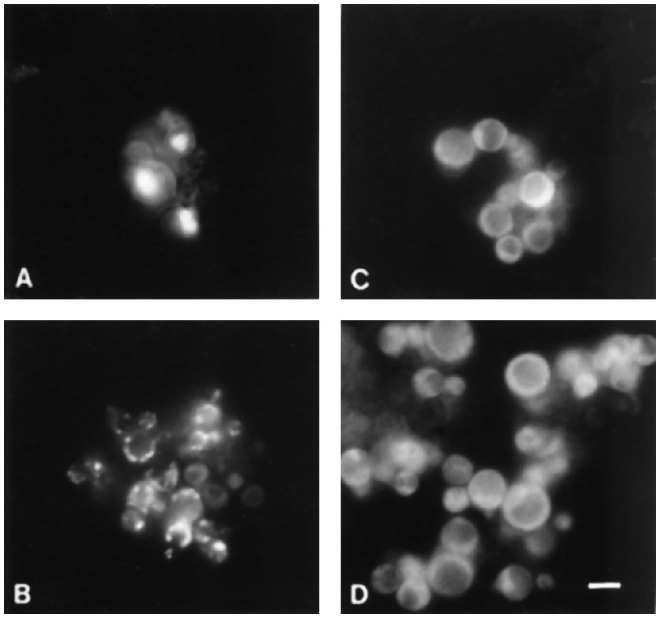

FIG. 4. **Detection of GFP-PTS1 and PTS2-GFP in wild type and null mutant cells.** The subcellular localization of the GFP-PTS1 and GFP-PTS2 reporter proteins was examined in living cells by fluorescence microscopy. Wild type strain STW1 (*panel A*) and mutant strain SEW2 (*panel C*), both expressing GFP-PTS1, were induced for 20 h in methanol medium. Wild type strain STW2 (*panel B*) and mutant strain SEW3 (*panel D*), both expressing PTS2-GFP, were induced for 20 h in oleate medium. *Bar* = 1 μm.

Peroxisomal Remnants Are Not Detectable in the Pppas2 Null Mutant—Ultrastructural examination of the null mutant did not reveal any morphologically distinguishable peroxisomes on methanol (Fig. 5*C*) or oleate (results not shown). In wild type cells, the peroxisomal compartment was clearly visible and had a characteristic morphology when grown in methanol (Fig. 5*A*) and oleic acid (Fig. 5*B*). Large, clustered peroxisomes filled with alcohol oxidase were seen in methanol-grown cells; and small, more abundant peroxisomes, identified as such by the presence of 3-ketoacyl-CoA thiolase, were evident in oleate-grown cells.

An enriched organelle fraction was prepared from wild type cells induced on methanol by differential centrifugation and subjected to isopycnic centrifugation on a continous Nycodenz gradient. Peroxisomes migrated to their buoyant density of 1.23 g/ml (fractions 2–6) as judged by the distribution of catalase (Fig. 6*A*), well separated from the bulk of mitochondria (fractions 12–20) identified by cytochrome *c* oxidase. Organelles from the null mutant, fractionated in the same way, lacked normal peroxisomes, whereas mitochondria were found at their normal position in the gradient (Fig. 6*C*). Only a small amount of catalase activity was recovered in organellar fractions of lower density in the Nycodenz gradient.

Equal portions of the gradient fractions from both wild type and null mutant cells were analyzed by immunoblotting. Alcohol oxidase, PpPas8p, and PpPas2p were predominantly peroxisomal (Fig. 6*B*), with some trailing of PpPas2p and PpPas8p into the lighter parts of the gradient, a phenomenon observed frequently with membrane-associated proteins. In the gradient from the null mutant, none of these proteins were detected in the denser parts of the gradient where peroxisomes usually band (Fig. 6, *C* and *D*). However, as observed in the wild type, traces of PpPas8p were found in gradient fractions of lower density in the null mutant (Fig. 6, *B* and *D*).

PpPas2p Is a Peroxisomal Membrane Protein—The location

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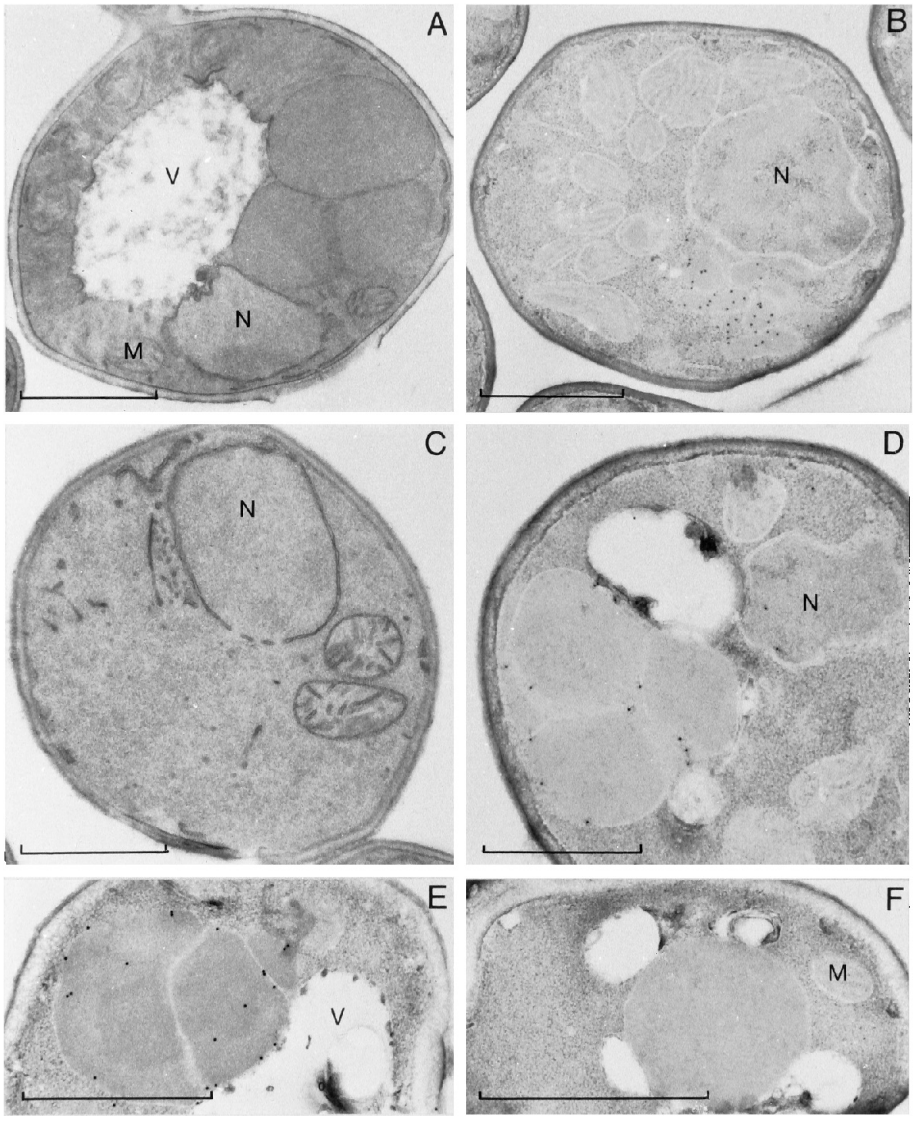

FIG. 5. **Ultrastructural analysis of wild type and null mutant cells.** Sections of $KMnO_4$-fixed, methanol-grown cells of wild type PPY1 (*panel A*) and the null mutant (*panel C*) are shown. *Panel B*, immunocytochemical detection of the β-oxidation enzyme 3-ketoacyl-CoA thiolase using anti-ScFox3p in a section of *p*-formaldehyde-fixed, oleic acid-grown cells of PPY1. Note that the morphological appearance of the peroxisomal compartment differs, with a few large clustered peroxisomes in methanol-induced cells (*panel A*) and smaller, more abundant, and dispersed peroxisomes in oleate-induced cells (*panel B*). No peroxisomal structures are visible in the null mutant (*panel C*). The vesicles adjacent to the nuclear membrane are also observed routinely in wild type *P. pastoris* cells (Gould *et al.*, 1992) and are not thought to be of peroxisomal origin. *Panels D* and *E*, immunocytochemical detection of PpPas2p in methanol-grown cells using anti-PpPas2p or the appropriate preimmune serum as the control (*panel F*). *N*, nucleus; *V*, vacuole; *M*, mitochondrion. *Bar*, 0.5 μm.

of PpPas2p within the peroxisomes was analyzed by immunocytochemistry and organelle subfractionation. Sections of methanol-grown wild type cells were incubated with anti-PpPas2p followed by gold-conjugated protein A. The gold particles almost exclusively decorated the peroxisomal membrane (Fig. 5, *D* and *E*). A control incubation using the preimmune antiserum showed no labeling of the peroxisomal membrane (Fig. 5*F*).

Strain STW1 was grown in SM medium, and crude organelle pellets were fractionated into soluble and insoluble fractions after treatment with 10 mM Tris, pH 8.5 (low salt), 1 M NaCl in 10 mM Tris, pH 8.5 (high salt), or 0.1 M Na_2CO_3 as described under "Materials and Methods." PpPas2p, PpPas8p, and PpPer6p were insoluble under all three conditions (Fig. 7*A*), thus behaving like integral membrane proteins. Catalase, a marker for soluble matrix proteins, was found in the supernatant under all conditions tested.

The NH_2-terminal 40 Amino Acids of PpPas2p Contain a Peroxisomal Targeting Signal—Analysis of the PpPas2p amino acid sequence did not reveal any known PTS. Several NH_2-terminal fragments of PpPAS2p, varying in length from 40 to 110 amino acids, were tested for their ability to target the reporter protein GFP to peroxisomes. As shown in Fig. 8, the smallest hybrid protein, consisting of the NH_2-terminal 40 amino acids of PpPas2p fused to GFP (1–40-GFP), was properly targeted to peroxisomes.

To address the subperoxisomal localization of the 1–40-GFP fusion, crude organellar fractions were prepared from strains STW1 and SKF1 grown on SM medium, and the extractability of the fusion proteins after low salt, high salt, or carbonate treatment was analyzed by Western blotting. In strain STW1, the GFP-PTS1 fusion protein was completely soluble after carbonate treatment, and even after low and high salt extraction the bulk of the fusion protein was found in the supernatants as expected for soluble matrix proteins (Fig. 7*A*). In strain SKF1, however, the 1–40-GFP fusion protein was insoluble under all three conditions (Fig. 7*B*), behaving like the full-length PpPas2p, as well as PpPas8p and PpPer6p (Fig. 7*A*).

PpPas2p Is Homologous to S. cerevisiae Pas3p Protein—A data base search using the BLAST program revealed that the PpPas2p protein displays a high similarity to ScPas3p, a peroxisomal integral membrane protein (Höhfeld *et al.*, 1991) but not to other proteins. From the sequence alignment (Fig. 9*A*) an overall identity of 35% was determined. There is a significant similarity (62%) over the entire length of PpPas2p and ScPas3p, but a number of segments that are highly conserved can be distinguished (*e.g.* PpPas2p amino acid residues 49–64, 70–78, 215–233, and 442–453). The putative membrane-spanning domain (residues 18–39) and membrane-anchoring domain (residues 135–153) of ScPas3p do not seem to be partic-

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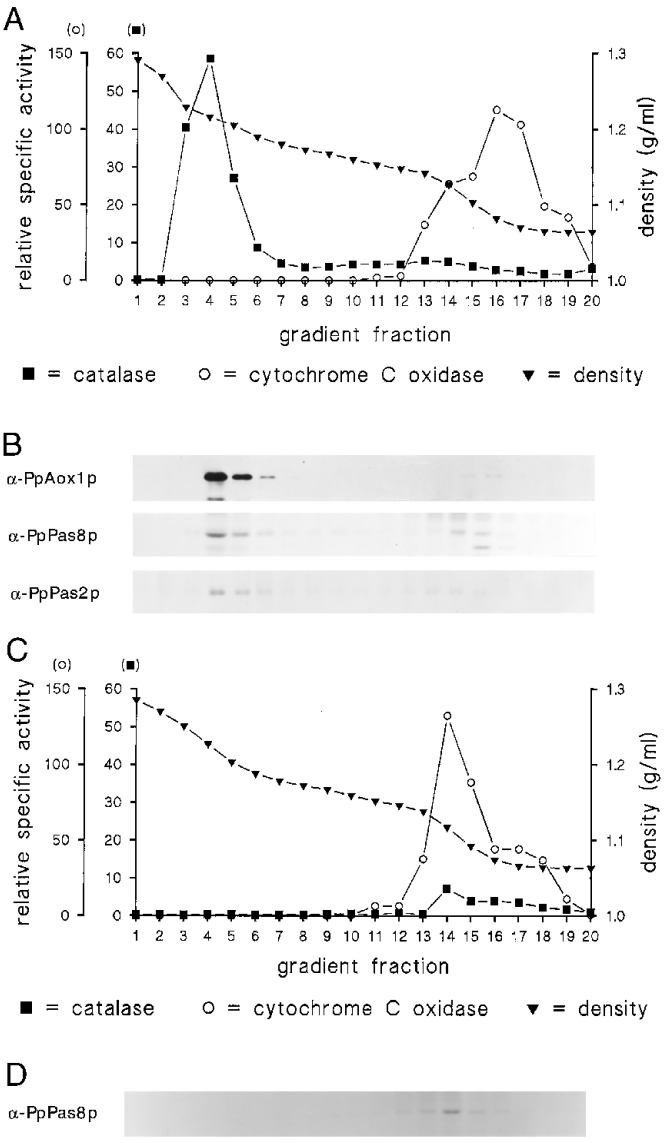

FIG. 6. **Nycodenz density gradient fractionation of organelles from wild type and null mutant cells**. Crude organelle fractions from methanol-induced cells, consisting primarily of peroxisomes and mitochondria, were fractionated on Nycodenz gradients as described under "Materials and Methods." The distribution of peroxisomal catalase and mitochondrial cytochrome *c* oxidase in gradients of wild type strain PPY4 (*panel A*) and null mutant SEW1 (*panel C*) is shown. Enzyme units are expressed as relative specific activities, reflecting the percentage enzyme activity in a given fraction relative to the total amount of protein contained in the gradient. Equal volumes of fractions from the gradients of wild type PPY4 (*panel B*) and the null mutant SEW1 (*panel D*) were analyzed by immunoblotting. Blots were incubated with antisera against PpPas8p (α-PpPas8p, dilution 1:5,000), PpPas2p (α-PpPas2p, dilution 1:5,000), and against alcohol oxidase (α-PpAox1p, dilution 1:20,000). No PpAox1p could be detected in gradient fractions of the null mutant.

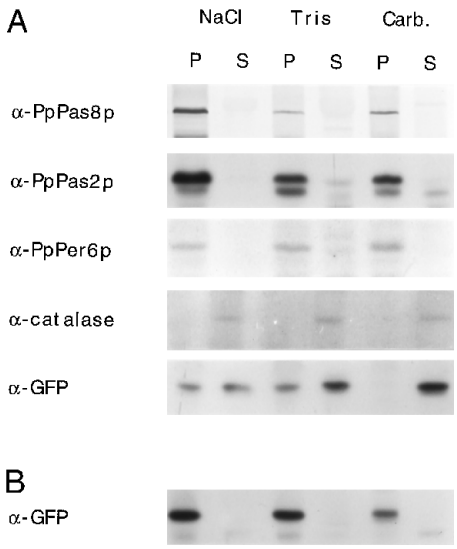

FIG. 7. **Subperoxisomal localization of PpPas2p and heterologous proteins in strains STW1 (GFP-PTS1) and SKF1 (1–40-GFP).** Crude organelle fractions were prepared from methanol-induced strains STW1 (*panel A*) and SKF1 (*panel B*) and subfractionated into an insoluble pellet fraction (*P*) and a soluble fraction (*S*) after treatment with NaCl, Tris, or Na_2CO_3 (*Carb.*) as described under "Materials and Methods." Distributions of PpPas2p, PpPas8p, PpPer6p, catalase, and the GFP fusion proteins between supernatant and membranous pellet fractions were examined by immunoblotting. The distribution of the four endogenous proteins was identical for both strains and is shown for strain STW1. Antibodies used were α-PpPas2p (dilution 1:5,000), α-PpPas8p (dilution 1:10,000), α-PpPer6p (dilution 1:2,000), α-catalase (dilution 1:5,000), and α-GFP (dilution 1:5,000).

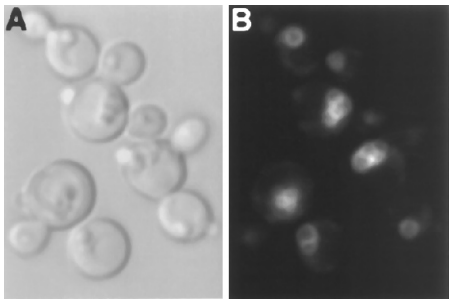

FIG. 8. **A PTS resides near the NH_2 terminus of PpPAS2p**. Localization of a hybrid protein consisting of the NH_2-terminal 40 amino acids of PpPAS2p and the GFP (1–40-GFP) was determined by confocal microscopy. *P. pastoris* cells producing the hybrid protein were grown in synthetic media containing methanol as sole carbon source. Note that the cells have large, clustered peroxisomes (*panel A*) which colocalize with the GFP fluorescence (*panel B*).

ularly conserved in PpPas2p. The hydrophobicity plot, prepared according to Kyte and Doolittle (1982), shows that the NH_2-terminal portion of PpPas2p is not as hydrophobic as the corresponding part of ScPas3p (Fig. 9*B*, compare with Fig. 5. in Höhfeld *et al.* (1991)). However, two hydrophobic domains seem to be formed by amino acid residues 66–79 and 149–174.

DISCUSSION

Most of the *P. pastoris pas* and *per* mutants characterized to date harbor morphologically and biochemically detectable peroxisome ghosts or membrane remnants (McCollum *et al.*, 1993; Spong and Subramani, 1993; Heyman *et al.*, 1994; Kalish *et al.*, 1995; Liu *et al.*, 1995) reminiscent of the structures seen in human patients suffering from generalized peroxisomal disorders. The *Pppas2* null mutant described in this study is an exception to this rule since it lacks morphologically detectable peroxisomes (Fig. 5*C*) even upon induction on methanol or oleate, both of which are known to induce peroxisomes. In cell fractionation experiments normal peroxisomes could not be detected either (Fig. 6, *C* and *D*). Traces of the peroxisomal membrane-associated protein PpPas8p, however, were found in fractions of lower density near the top of the gradient in both wild type (Fig. 6*B*) and the null mutant (Fig. 6*D*). Whether this represents a real peroxisomal (precursor) fraction or nonspecific binding of PpPas8p to mitochondria or other membranous particles is unknown. In the *Pppas2* null mutant, marker proteins containing a PTS1 or a PTS2 sequence were found to be localized to the cytoplasm (Fig. 4). These data suggest that PpPas2p is involved in the early stages of peroxisome biogenesis. Therefore, we propose that the proper functioning of

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PpPas2p is a prerequisite for the assembly of matrix, and at least some membrane, proteins into peroxisomes.

PpPas2p is homologous to Pas3p of *S. cerevisiae* (ScPas3p), which has been shown by Höhfeld *et al.* (1991) to be a peroxisomal integral membrane protein. Immunocytochemical detection of PpPas2p revealed that it is also associated with the peroxisomal membrane (Fig. 5, *D* and *E*). This was further substantiated by biochemical experiments that showed that the protein behaves like an integral membrane protein during subfractionation of an organelle pellet fraction (Fig. 7).

Earlier experiments show that there are at least two different signals that target proteins to the peroxisomal matrix. PMPs must use a different targeting signal, which remains to be defined. Proteins that reside in the peroxisomal membrane can be envisaged as having two components: an mPTS, which targets the protein to the peroxisome, and a transmembrane domain (TMD), which anchors the protein in the membrane, or a protein-protein interaction domain that anchors it to the membrane through other peroxisomal integral membrane proteins. These two components could either be separate or overlapping.

The PTS in ScPas3p has been described to lie in the NH_2-terminal half of the protein, a region that encompasses a putative transmembrane domain (Höhfeld *et al.*, 1992). Our result on the targeting of the 1–40-GFP fusion reveals the presence of an mPTS in the NH_2-terminal 40 amino acids of PpPas2p (Fig. 8). In contrast to ScPas3p, the first 40 amino acids of PpPas2p do not contain any obvious TMD (Figs. 1 and 9*B*). Yet the 1–40-GFP fusion is directed faithfully to the peroxisomal membrane, where it behaves exactly like the full-length PpPas2p in terms of its inextractability with sodium carbonate (Fig. 7). This suggests that these 40 amino acids have both an mPTS as well as a domain that allows the fusion to interact tightly with the peroxisomal membrane or with one or more PMPs. The sequence and experimental data seem contradictory, having a protein behaving like an integral membrane protein without predicted transmembrane segments. On one hand, several (peroxisomal) proteins have been shown to be sodium carbonate-inextractable (Tan *et al.*, 1995; Erdmann and Blobel, 1995) from the membranes, whereas no clear transmembrane segments could be detected by sequence analysis. On the other hand, some proteins with strong protein-protein interactions might resist the sodium carbonate extraction procedure and therefore are falsely classified as integral membrane proteins. Based on the relatively low content of hydrophobic residues and the presence of a highly positively charged domain in the NH_2-terminal 40 amino acids, we favor the possibility of a strong interaction between this sequence in PpPas2p and another peroxisomal integral membrane protein.

Based on the proposed topology of ScPAS3p, we expected that the GFP segment in the 1–40-GFP hybrid protein would face the cytosol. Therefore, we performed a proteinase K protection experiment on a crude organellar fraction to determine whether it would be sensitive to proteolytic degradation. The hybrid protein appeared to be resistant to proteolytic degradation, whether or not the organelles were disrupted by Triton X-100 prior to the proteinase K treatment. This suggests that GFP itself is highly resistant to proteolytic degradation, and therefore the results were not conclusive.

A putative mPTS has been identified in a *Candida boidinii* protein, CbPMP47. This protein is homologous to a family of mitochondrial solute transporters that span the membrane six times (Kuan and Saier, 1993). The targeting information on this protein was localized to a region of the protein containing transmembrane domains 4 and 5 and an intervening 20-amino acid loop facing the peroxisomal matrix (McCammon *et al.*, 1994). In a more detailed analysis of the targeting information in CbPMP47 it was shown that a stretch of 20 amino acids in the intervening loop between transmembrane domains 4 and 5 was able to direct a soluble reporter protein to the peroxisomal membrane of *S. cerevisiae* (McNew and Goodman, 1996). It is interesting to note that in both PpPas2p and in CbPMP47 the mPTS does not include any predicted TMD. This supports the idea proposed above that the mPTS and TMD are separable entities and that it is the mPTS that directs a TMD in a protein

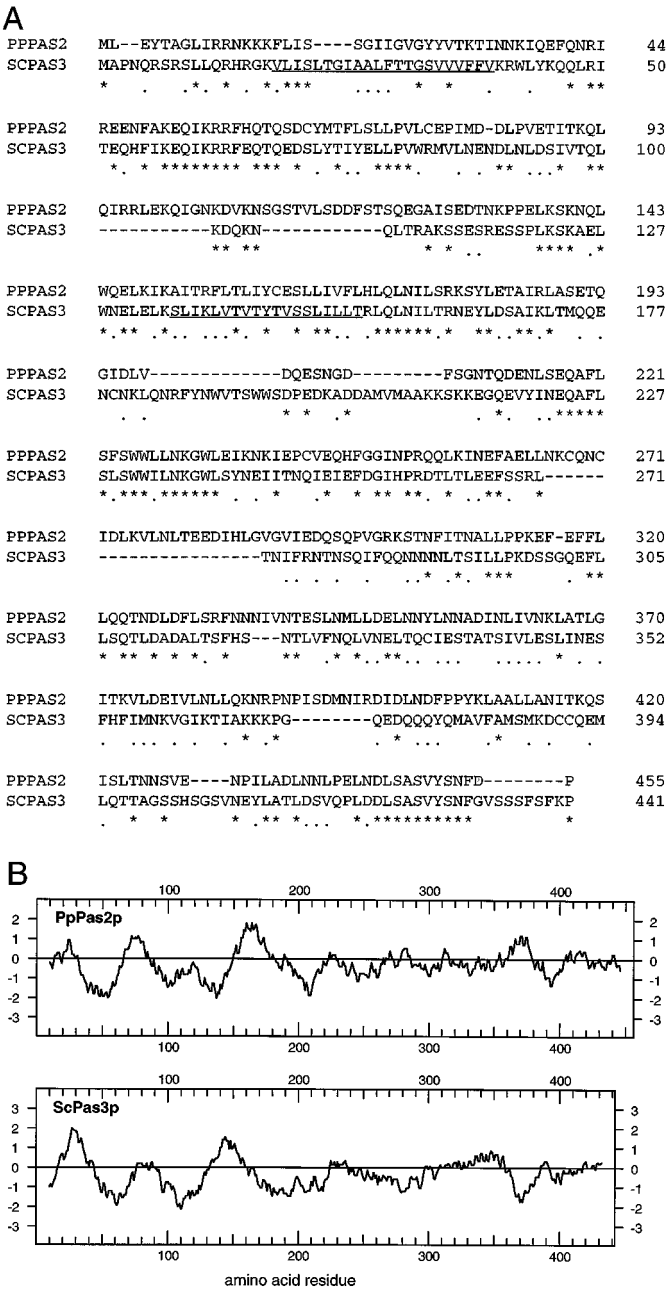

FIG. 9. **Sequence comparison and hydrophobicity analysis of PpPas2p and ScPas3p.** The amino acid sequences were aligned using the BESTFIT program (*panel A*). Identical residues are indicated by an *asterisk* below the sequence; a *dot* represents aligned residues that are similar. Two hydrophobic domains, identified by Höhfeld *et al.* (1991), in ScPas3p are *underlined*. Similarity rules: G = A = S; V = I = L = M = F = Y = W; K = *r* = H; D = E = Q = N; S = T = Q = N. *Panel B*, hydrophobicity plot of PpPas2p and ScPas3p according to Kyte and Doolittle (1982), using a window size of 19 amino acids. Two hydrophobic domains are revealed in PpPas2p, a 13-amino acid stretch at position 66–79, and a potential membrane-spanning region at position 149–174.

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to the peroxisomal membrane. This model predicts that the TMDs of PMPs would have no higher affinity for the peroxisomal membrane relative to other subcellular membranes.

What are the features common to mPTSs from different proteins? A comparison of the 20-amino acid segment of CbPMP47 and the NH_2 termini of PpPas2p and ScPas3p reveals a block of five amino acids (of which four are positively charged) in CbPMP47 which is also observed in both PpPas2p and ScPas3p (QIKRR) at positions 53–57 and 59–63, respectively, being 100% conserved between these Pas proteins. Notably, this sequence is excluded from the first 40 amino acids of PpPas2p, which are able to direct GFP to the peroxisomal membrane. Another positively charged sequence (RRNKKK) present at position 10–15 might be involved in targeting of PpPas2p, although this sequence is not particularly conserved in ScPas3p.

Mutational analysis of the first 40 amino acids of PpPas2p will reveal whether the mPTS and peroxisomal membrane interaction domain are overlapping or physically separable entities. It may also help address whether an mPTS and peroxisomal membrane interaction domain need to coexist for proper targeting to the peroxisomal membrane.

Studies on the import of PMPs into peroxisomes *in vitro* have been undertaken without any knowledge of the mPTSs involved in the targeting process (Diestelkotter and Just, 1993; Imanaka *et al.*, 1996). These experiments reveal that import of such proteins is dependent on time and temperature, does not require ATP or GTP, and is not inhibited by *N*-ethylmaleimide treatment of either the soluble components or of the isolated peroxisomes. In contrast, the import of the peroxisomal matrix marker, firefly luciferase, into peroxisomes of permeabilized cells is dependent on ATP hydrolysis and is blocked by *N*-ethylmaleimide pretreatment of the cytosol-depleted cells (Rapp *et al.*, 1993; Wendland and Subramani, 1993). These properties, as well as the import of PMPs into peroxisome ghosts in yeast and human cell lines deficient in the import of PTS1- and PTS2-containing proteins, imply that the mechanism of import of PMPs is different from that of the matrix proteins. The identification of a membrane targeting signal in PpPas2p should aid in the identification of the receptors for the mPTS and in the definition of the other components required for the targeting of PMPs.

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