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 Ppas2 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 Ppas2 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$_2$O$_2$ metabolism and the $\beta$-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 $\beta$-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., 1993; 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; Molley et al., 1994; Marzioch et al., 1994; Slaeste 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 ScPasb1p) 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; Creg 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).
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 μM 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. Matting, 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-PASP2B. A set of plasmids with nested deletions generated by exonucleaseIII and S1 nuclease (Erase-A-Base kit, Promega, Madison, WI) was used for sequencing both DNA strands.

Construction of the PpPas2 Null Mutant—An EcoRI-Acd fragment of 1,230 base pairs (nucleotides 116-1346), encompassing most of the PpPas2 coding region, was replaced by a 2.0-kb EcoRI-HindIII fragment containing the P. pastoris ARG4 gene (see Fig. 2). The Acd and HindIII sites were filled in using Klenow polymerase. Plasmid PpHs4 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 Smal fragment (polylinker sites) into the EcoRI site (blunted with Klenow polymerase) of pJ AH35, a pBR322-based vector containing the PpHis4 gene and a P. pastoris autonomously replicating sequence (PARS2, Cregg et al., 1985).

Plasmid pTW66, containing the green fluorescent protein-PTS1 (GFP-PTS1) construct under control of the methanol-inducible alcohol oxidase promoter (PaOX1) with the addition of an EcoRI site (blunted with Klenow polymerase) (PpHs4, given by Dr. W. H. Kunau (Ruhr University, Bochum, Germany). Plasmid pTW66, containing the PTS2-GFP construct under control of the constitutive glyceraldehyde 3-phosphate dehydrogenase promoter (PpGAPDH), a gift of Dr. M. Cregg, Oregon Graduate Institute of Science and Technology, Portland, OR) was constructed by cloning the GFP-PTS1 allele in-frame with a part of the Sf cx3 sequence (Glover et al., 1994) encoding the N-terminal 16 amino acids of the Saccharomyces cerevisiae 3-oxoacyl-CoA thioesterase in vector pHIL-2 (Dixon, 1991).

A hybrid gene encoding the N-terminal 40 amino acids of PpPas2p and GFP (1–40-GFP) was constructed as follows. By PCR, an Asp-718 and a BglII site were introduced upstream of the PpPas2 open reading frame present in pBSII KS, resulting in pKNSD44. A second PCR was performed to amplify a DNA fragment encoding the GFP-PTS1 allele flanked by an EcoRI (5′) and a Spe (3′) site which was inserted into EcoRI and Spe-digested pKNSD44 resulting in pKNSD73. Finally, the hybrid gene was inserted as a 0.9-kb BglII-NotI DNA fragment into the polylinker of pIFC3K (Invitrogen), resulting in pKNSD77. All of the GFP expression vectors were linearized by Sall 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 oxidase activities were determined as described by Baadhuisen et al. (1994) 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 (Amerham Corp.) after incubation of blots with a protein A-horseradish peroxidase conjugate (dilution 1:3,000, Bio-Rad).

Antibodies—An Acd (filled in with Klenow polymerase)-HindIII fragment of 1,717 base pairs (amino acids 8–455) of PpPas2A was cloned between the XbaI (filled in with Klenow polymerase) and HindIII sites of the pGEK-KG polylinker (Guan and Dixon, 1991). The resulting glutathione S-transferase PpPas2 fusion protein was synthesized in E. coli DH5α as described by Guan and Dixon (1991), except that no benzamide was used, and the cells were lysed by sonication. The largely insoluble GST-PpPas2 fusion protein was isolated by SDS-PAGE and cleaved by thrombin. The 52-kDa GST-PpPas2 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-Sfcox3p (anti-S. cerevisiae 3-ketoacyl-CoA thioesterase) antibodies were a gift from Dr. W. H. Kunau (Ruhr University, Bochum, Germany). Anti-PpPas2p, 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.
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 MgCl₂, 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 2°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 mM NaCl, 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 fusion proteins were analyzed by SDS-PAGE and immunoblotting.

**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).

**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.
PpPas2p Is a Peroxisomal Membrane Protein—The location with the PpARG4 gene (Fig. 2). The resulting PpPas2p 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 PpPas2p null mutant and the PpPas2p 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. Introduction of PpPas2p 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 PpPas2p 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. 4A). These results are in agreement with data published by Monosov et al. (1996). Likewise, a hybrid protein consisting of the NH₂-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. 4B).

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. 4C) as well as PTS2-GFP (Fig. 4D) were present in the cytoplasm as indicated by the diffuse fluorescent signal.

Peroxisomal Remnants Are Not Detectable in the PpPas2p Null Mutant—Ultrastructural examination of the null mutant did not reveal any morphologically distinguishable peroxisomes on methanol (Fig. 5C) 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. 5A) or oleic acid (Fig. 5B). 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 continuous Nycodenz gradient. Peroxisomes migrated to their buoyant density of 1.23 g/ml (fractions 2–6) as judged by the distribution of catalase (Fig. 6A), 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. 6C). Only a small amount of catalase activity was recovered in organelar 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. 6B), 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
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 Na2CO3 as described under “Materials and Methods.” PpPas2p, PpPas8p, and PpPer6p were insoluble under all three conditions (Fig. 7A), thus behaving like the full-length PpPas2p, as well as PpPas8p and PpPer6p (Fig. 7A).

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. 9A) 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-
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. 9B, 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. 5C) 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. 6B) and the null mutant (Fig. 6D). 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...
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 9B). 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 proteasome K protection experiment on a crude organelle 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 proteasome 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 (McCann 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 = L = M = F = Y = W; K = R = H; D = E = Q = N; S = T = O = 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.
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 mPTTs from different proteins? A comparison of the 20-amino acid segment of Cb-mal membrane relative to other subcellular membranes. TMDs of PMPs would have no higher affinity for the peroxisomal membrane. This model predicts that the mPTS and peroxisomal membrane interaction domain need to coexist for proper targeting to the peroxisomal membrane.

Studies on the import of PMPs into peroxisomes have been undertaken without any knowledge of the mPTTs involved in the targeting process (Diestelkotter and J ust, 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.

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 properties, as well as the import of PMPs into peroxisomes, are dependent on ATP hydrolysis and is blocked by N-ethylmaleimide pretreatment of the cytosol-depleted cells.