The *pas8* Mutant of *Pichia pastoris* Exhibits the Peroxisomal Protein Import Deficiencies of Zellweger Syndrome Cells—The PAS8 Protein Binds to the COOH-Terminal Tripeptide Peroxisomal Targeting Signal, and Is a Member of the TPR Protein Family

Dannel McCollum, Edward Monosov, and Suresh Subramani

Department of Biology, University of California at San Diego, La Jolla, California 92093-0322

Abstract. We previously described the isolation of mutants of the yeast *Pichia pastoris* that are deficient in peroxisome assembly (*pas* mutants). We describe the characterization of one of these mutants, *pas8*, and the cloning of the *PAS8* gene. The *pas8* mutant is deficient for growth, but not for division or segregation of peroxisomes, or for induction of peroxisomal proteins. Two distinct peroxisomal targeting signals, PTS1 and PTS2, have been identified that are sufficient to direct proteins to the peroxisomal matrix. We show that the *pas8* mutant is deficient in the import of proteins with the PTS1, but not the PTS2, targeting signal. This is the same import deficiency as that found in cells from patients with the lethal human peroxisomal disorder Zellweger syndrome. Cloning and sequencing of the *PAS8* gene reveals that it is a novel member of the tetratricopeptide repeat gene family. Antibodies raised against bacterially expressed PAS8 are used to show that PAS8 is a peroxisomal, membrane-associated protein. Also, we have found that in vitro translated PAS8 protein is capable of binding the PTS1 targeting signal specifically, raising the possibility that PAS8 is a PTS1 receptor.

THE compartmentalization of cellular processes into different subcellular compartments is one of the hallmarks of the eukaryotic cell. One such compartment, the peroxisome, is ubiquitously present in virtually all eukaryotic cells. Specialized versions of peroxisome-like compartments (e.g., glycosomes of trypanosomes and glyoxysomes of plants) exist in certain organisms. The peroxisomes, glycosomes, and glyoxysomes have been termed collectively as microbodies. Peroxisomes are characterized by the presence of at least one peroxide-generating oxidase and the enzyme catalase which degrades hydrogen peroxide. Peroxisomes are bounded by a single membrane and vary in size from 0.1 to 1.0 μm in diameter. Like mitochondria, peroxisomes are thought to arise by budding and division of preexisting organelles. Unlike mitochondria, peroxisomes do not contain DNA and must import all constituent proteins. Both peroxisomal matrix and membrane proteins are synthesized in the cytosol on free polysomes and imported posttranslationally into the organelle (for review see Lazarow and Fujiki, 1985).

Although the targeting signals for peroxisomal membrane proteins are unknown, recent work has demonstrated the existence of at least two distinct signals that will direct proteins to the peroxisomal matrix (for review see Subramani, 1992). The most common peroxisomal targeting signal (PTS1)[1] is typified by the carboxy-terminal signal SKL found in the firefly luciferase protein. Certain conservative substitutions in this signal are allowed in mammalian cells (Gould et al., 1989; Swinkels et al., 1992) and in yeast (Aitchison et al., 1991). The SKL signal can target proteins to microbodies in virtually all eukaryotes from trypanosomes, fungi, and plants to mammals (Gould et al., 1990). Antibodies made against the SKL peptide also recognize microbodies in all eukaryotic species that have been examined (Keller et al., 1991). These results demonstrate that the mechanisms of peroxisomal protein import are likely to be conserved among all eukaryotes.

A second peroxisomal targeting signal (PTS2) has been found near the amino terminus of the rat peroxisomal 3-ketoacyl CoA thiolase protein. This signal has been delimited to an 11-amino acid sequence that bears no similarity to the PTS1 signal. Like PTS1, PTS2 can direct heterologous proteins to the peroxisome. Little is known about the evolutionary conservation of PTS2, although it appears to be conserved in several fungal thiolases (Swinkels et al., 1991;

Please address all correspondence to Dr. S. Subramani, Department of Biology, 0322 Bonner Hall, Room 3226, University of California San Diego, La Jolla, CA 92093.

1. *Abbreviations used in this paper*: DBS, dilution of blocking solution; DHAS, dihydroxyacetone synthase; HSA, human serum albumin; MW, molecular weight; Mut, methanol utilization; PAS, peroxisome assembly; PTS, peroxisome targeting signal; SD, synthetic dextrose; SM, synthetic methanol; SOL, synthetic oleate; SOLT, synthetic oleate and Tween; TPR, tetratricopeptide repeat; YP, yeast extract peptone; YPD, yeast extract peptone dextrose; YPM, yeast extract peptone methanol; YPOLT, yeast extract peptone oleate Tween.

Osumi et al., 1991). It also remains to be determined whether other peroxisomal proteins besides thiolase use the PTS2 targeting signal.

The importance of peroxisomes in human development is demonstrated by the lethal genetic disorder Zellweger syndrome (for review see Lazarow and Moser, 1989). Zellweger patients possess defects in numerous peroxisomal pathways and usually die shortly after birth (Zellweger, 1988). Initially it was believed that Zellweger patients completely lacked peroxisomes because peroxisomal matrix proteins were mislocalized to the cytosol. Upon further examination, cells from these patients were found to contain "peroxisome ghosts" that have peroxisomal membrane proteins but do not contain most matrix proteins (Santos et al., 1988*a*,*b*). Although, as Zellweger syndrome illustrates, peroxisomes are essential for viability in humans, they are not essential at the cellular level. Because of this, peroxisomes are ideally suited for the investigation of the little-studied, but fundamentally important, problem of organelle biogenesis. In certain yeast species, peroxisomes, unlike other organelles, can be dispensed with under specific nutritional conditions. This facilitates the isolation of mutants in peroxisome assembly and import without compromising the viability of the cell. Peroxisomes also offer a unique opportunity to study the biochemistry and genetics of intracellular organelle disassembly and degradation because the organelle can be made to proliferate in response to specific nutritional cues or to undergo degradation in a different environmental milieu. Mutants defective in peroxisome assembly have been isolated in CHO cells (Zoeller and Raetz, 1986; Tsukamoto et al., 1990), and in the three yeast species *Hansenula polymorpha* (Cregg et al., 1990; Didion et al., 1990), *Saccharomyces cerevisiae* (Erdmann et al., 1989; van Der Leij et al., 1992), and *Pichia pastoris* (Gould et al., 1992; Liu et al., 1992). These mutants have allowed the cloning of one *PAS* gene from CHO cells (Tsukamoto et al., 1991), and three *PAS* genes from *S. cerevisiae* (Erdman et al., 1991; Hohfeld et al., 1991; Wiebel and Kunau, 1992). These genes clearly play an essential role in peroxisome formation, but at present it is uncertain whether they are involved in protein import or some other aspect of peroxisome assembly or function.

Studies involving a genetic analysis of peroxisome biogenesis and research on peroxisomal targeting signals are beginning to converge. The discovery of the amino-terminal targeting signal of rat thiolase predicts that there must be at least two distinct pathways involved in peroxisomal protein import. Genetic evidence supports this hypothesis. Some Zellweger patients are unable to import proteins containing the PTS1 signal (Walton et al., 1992), but do import thiolase into their peroxisomes (Balfe et al., 1990). This suggests that the defect in at least some Zellweger patients is in the SKL-dependent protein import pathway. Conversely, a *S. cerevisiae* mutant has been isolated that is defective in the import of thiolase, but not other peroxisomal proteins (Kunau and Hartig, 1992; van Der Leij et al., 1992). A third class of mutants has been isolated in yeast that appears to mislocalize all peroxisomal matrix proteins analyzed to the cytosol (Erdmann et al., 1989; Hohfeld et al., 1991; Cregg et al., 1990). It remains to be determined whether this mislocalization of all peroxisomal proteins represents a true defect in peroxisomal protein import or is a secondary consequence of a more general defect in peroxisome assembly. Indeed, it is not clear whether this latter class of mutants has any peroxisomes. In this paper we show that the *pas8* mutant of *P. pastoris* can import thiolase into peroxisomes, but is defective in the PTS1 import pathway. This yeast mutant has the same phenotype as Zellweger syndrome cells, and represents a good model system for the study of Zellweger syndrome. It was recently reported that the *pas10* mutant of *S. cerevisiae* imports thiolase, but not catalase (van Der Leij et al., 1992), which suggests that it may have the same phenotype as the *P. pastoris pas8* mutant. *PAS8* is also the first *PAS* gene reported to play an essential role in peroxisomal protein import.

Materials and Methods

Strains, Culture, and Microbial Manipulations

All genetic manipulations and *P. pastoris* strains are described previously (Gould et al., 1992). *P. pastoris* cells were grown on either minimal synthetic media (S) consisting of 6.7 g/l yeast nitrogen base (Difco Laboratories Inc., Detroit, MI), supplemented with carbon sources to a final concentration of either 2% synthetic dextrose (SD), 0.5% synthetic methanol (SM), 2% synthetic ethanol (SE), 0.2% oleic acid synthetic oleate (SOL), or 0.2% oleic acid/0.02% Tween-40 synthetic oleate and Tween (SOLT). Amino acids, uracil, and adenine were added to a final concentration of 40 μg/ml. Standard rich medium for growth of *P. pastoris* was YP medium (1% yeast extract, 2% bacto-peptone) supplemented with carbon sources to final concentrations of 2% dextrose (YPD), 0.5% methanol (YPM), or 0.2% oleic acid/0.02% Tween-40 (YPOLT). *P. pastoris* cells were routinely cultured at 30°C. Yeast transformations were by the spheroplast method (Cregg et al., 1985) or electroporation (Rickey, 1990). Recombinant DNA techniques were used as described (Sambrook et al., 1989) using *E. coli* strain DH5αF' (GIBCO-BRL, Gaithersburg, MD). Miniprep DNA samples were prepared using a modified alkaline lysis procedure (Zhou et al., 1990). Bacterial transformations were done as described (Chung et al., 1989). Whole cell extracts of *P. pastoris* were prepared as described (Tschopp et al., 1987).

Electron Microscopy

Wild-type and mutant *P. pastoris* cells to be used for morphological studies were prepared for EM as described previously (Gould et al., 1992; Luft, 1961). Cells were prepared for immunocryoelectron microscopy by first fixing them in a solution containing 4.0% freshly depolymerized paraformaldehyde, and 0.2% glutaraldehyde in 125 mM Hepes pH 7.2, for 1.5 h at room temperature. After fixation, cells were washed three times in 125 mM Hepes, pH 7.2, 0.002% glycine. Cells were then pelleted and pellets were infused with a mixture that contained 2.0% sucrose and 31.0% polyvinylpyrrolidone molecular weight (M.W. 10,000) in the Hepes buffer for 5 h at room temperature. Small portions of the pellets were frozen on aluminum rods in liquid freon followed by liquid nitrogen in which they were also stored. Ultrathin frozen sections were cut with glass knives on a MT-7 cryoultramicrotome (Research Manufacturing Co., Tucson, AZ). "Gold colored" sections were transferred to collodion-covered, carbon-coated grids. Sections were blocked by normal goat serum diluted (1:20) with 125 mM Hepes, pH 7.2, for 20 min at room temperature, and then rinsed with a tenfold dilution of blocking solution (DBS), followed by incubation with primary antibodies for 30 min at room temperature. After several washes in DBS, sections were incubated with secondary antibodies (goat anti-rabbit-gold conjugates) for 30 min at room temperature. After labeling, the sections were rinsed five times with DBS followed by five changes of boiled distilled water. Finally sections were stained and embedded in a solution containing 0.1% methyl cellulose and 0.3% phosphomolybdic acid (MW 3939.5). Phosphomolybdic acid was neutralized by 5 M NaOH to pH 5.3. Affinity-purified anti-methanol oxidase antibody (a gift from John Heyman) was further purified by adsorption with YPD-grown *P. pastoris* cell walls to remove reactivity to cell walls. Labeling for luciferase was performed using an IgG fraction prepared from guinea pig anti-luciferase serum (Harlow and Lane, 1988).

Fractionation of Peroxisomes

The localization of peroxisomal enzymes in wild-type and mutant cells was assessed using a differential centrifugation assay as described previously

(Gould et al., 1992) with the following modifications. Cells containing plasmids were first grown in SD medium to mid-log phase, and then shifted to rich inducing media (YPM or YPOLT), and incubated overnight. Cells that did not contain plasmids were grown as usual on YPD medium, and then were shifted to YPM or YPOLT. Induction of cells in rich inducing medium was found to make cells much easier to spheroplast. Enzyme assays for catalase (Leighton et al., 1968) and luciferase (De Wet et al., 1987) were used to determine their abundance in the different fractions. Thiolase and DHAS were analyzed by Western blotting (Harlow and Lane, 1988), using anti-thiolase (a gift from Wolf Kunau, Ruhr Univ., Bochum), and anti-DHAS (a gift from Joel Goodman, Univ. Texas Southwestern Med. Center, Dallas) antibodies. Purified peroxisomes were obtained by loading the organelle pellets of YPOLT-induced cells from the differential centrifugation assay on a Nycodenz gradient as previously described (Nuttley et al., 1990). Fractions were collected and assayed for catalase activity to determine the location of peroxisomes for wild-type cells. Because *pas8* peroxisomes do not import catalase but do import thiolase, gradient fractions from *pas8* cells were analyzed by Western blotting for the presence of thiolase. Peroxisomal fractions from both wild-type and *pas8* cells were found to be essentially free of mitochondrial contamination as assayed by succinate dehydrogenase activity (Sottocasa et al., 1967). Purified peroxisomes from wild-type and *pas8* cells were run on 10% SDS polyacrylamide gels and silver-stained (Sambrook et al., 1989) to determine the protein composition of wild-type and *pas8* peroxisomes.

The intra-peroxisomal localization of PAS8 was assessed by first preparing an organelle pellet fraction from wild-type cells induced in methanol medium for 16–18 h, as described above. Portions of this pellet were suspended in 10 mM Tris, pH 8.5, 5 mM EDTA, which ruptures peroxisomes, or 10 mM Tris, pH 8.5, 5 mM EDTA, 1 M NaCl, incubated on ice for 1 h and spun at 100,000 *g* to obtain supernatant and membrane-pellet fractions. Equivalent portions of the resulting supernatants and pellets were loaded on 10% SDS polyacrylamide gels and were analyzed by Western blotting using PAS8 antibodies. These experiments were done in the presence of 5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 1 mM PMSF, and 0.21 mg/ml NaF.

Isolation of the P. pastoris PAS8 Gene

pas8.1 arg4 cells were transformed by the spheroplast method (Cregg et al., 1985) with a *P. pastoris* genomic library constructed in the *P. pastoris* replicating vector pSG464 (Gould et al., 1992), and Arg^+ colonies were selected for in SD top agar. After 3 d, top agar and colonies were scraped from each plate and washed five times in water after which the cells were plated out onto SM plates and incubated for 5 d until colonies appeared. Single Mut^+ colonies were transferred to 20 ml of SM media and grown to saturation. These cells were harvested and their DNA was isolated as described (Ausubel et al., 1987) and used to transform *E. coli*. Ampicillin-resistant colonies were all found to contain the same plasmid designated p2–18. Plasmid p2–18 contained a 5.0-kb insert and was found, upon reintroduction into *pas8* cells, to fully complement the mutation. The complementing region of p2–18 was narrowed down to a 2.3-kb HindIII–SpeI fragment from which subclones were generated for sequencing. A 1.8-kb PstI–EcoRI fragment was cloned into pKS II (Stratagene, La Jolla, CA) for in vitro transcription and translation (Promega Corp., Madison, WI).

Disruption of the PAS8 Gene

The NcoI–NsiI fragment of p2–18 containing most of the *PAS8* open reading frame was replaced, using blunt ended cloning, with a 2-kb EcoRI–KpnI fragment of pYM32 containing the *P. pastoris ARG4* gene (NRRL B-18016). The HindIII fragment containing the *ARG4* gene and *PAS8* flanking region was isolated and transformed by electroporation into *arg4, his4 P. pastoris*. The resulting Arg^+, Mut^- transformants were crossed with the original *pas8* mutation and the resulting diploids were unable to grow on methanol confirming that the cloned gene corresponds to the *PAS8* gene.

Production of Anti-PAS8 Antibodies

Antibodies against the PAS8 protein were raised against a β-galactosidase–PAS8 fusion protein. Specifically, a 2.1-kb BamHI–HindIII fragment containing the carboxy-terminal 1409-bp of the 1729-bp *PAS8* gene was isolated from plasmid p2–18 and inserted into BamHI–HindIII cut vector pTRBO (Burglin and DeRobertis, 1987). The fusion protein was expressed in *E. coli* strain BB3, purified by SDS-PAGE and electroelution, and used to immunize rabbits (Harlow and Lane, 1988). PAS8 antiserum was diluted 1:200 for all Western blotting experiments.

PAS8 Binding Assays

The SKL peptide (CRYHLKPLQSKL) and the ΔSKL peptide (CRYHLKPLQ) were coupled to human serum albumin (HSA) using a heterobifunctional crosslinker to couple the amino groups on the lysine side chains of HSA to the cysteinyl sulfhydryl on the peptides, as described in Walton et al. (1992). HSA-peptide conjugates and the peptides were coupled to Affigel 15 and Affigel 102 (Biorad Labs., Hercules, CA), respectively, according to the manufacturer's specifications. 15 μl of in vitro translated PAS8 protein were mixed with 35 μl of binding buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EDTA, and 2 mM DTT), which was then added to a 25-μl vol of bead conjugate and incubated at 25°C for 1 h with frequent mixing. The supernatant, containing unbound PAS8 protein, was then removed and saved. The beads were washed four times each with 1 ml of binding buffer, and then suspended in 50 μl of binding buffer. Sample buffer was added to the fractions containing bound (beads) and unbound PAS8, in which they were boiled and one half of each sample was run on SDS polyacrylamide gels for fluorography. All bead conjugates were kindly provided by Dr. Martin Wendland. All solutions used in the binding assay contained protease inhibitors (5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.21 mg/ml NaF, and 1 mM PMSF).

Results

pas8 Cells Are Defective for Growth but Not Division of Peroxisomes

The advantages of the yeast *P. pastoris* for a genetic analysis of peroxisome biogenesis, and the isolation of a set of peroxisome assembly (*pas*) mutants in this organism have been described recently (Gould et al., 1992; Liu et al., 1992). During growth on glucose or ethanol as sole carbon source, *P. pastoris* cells contain only a few small peroxisomes. In contrast, incubation on either methanol or oleic acid causes a massive proliferation of peroxisomes. Previous work has shown that *pas* mutants of *P. pastoris* grow at rates similar to wild-type cells on glucose or ethanol, but are unable to grow on media containing methanol or oleic acid as sole carbon source. After incubation on methanol, *pas* mutants do not form normal peroxisomes, although in occasional sections one sees small flattened peroxisomal structures (Gould et al., 1992). A careful examination of *pas8* cells induced on methanol for 16–18 h revealed the frequent presence of clusters of small peroxisome-like structures in these cells Fig. 1 *B*, see *arrow*). Since *pas8* cells never displayed the enormous peroxisomes seen in wild-type cells (Fig. 1 *A*), it appears that *pas8* cells are capable of proliferating the peroxisome-like structures upon methanol induction but are deficient for growth of the organelle. To insure that the aberrant peroxisome-like structures found in *pas8* cells were not caused simply by the inability of the *pas8* mutant to grow on methanol, we also examined an *fdh* mutant which is deficient in the enzyme formaldehyde dehydrogenase. The formaldehyde dehydrogenase gene is essential for growth on methanol, but is not essential for peroxisome formation (Liu et al., 1992). After induction in methanol-containing medium, the *fdh* mutant was able to form normal peroxisomes (Fig. 1 *D*). Thus *pas8* cells clearly have a defect in peroxisome growth.

Because *pas* mutants are unable to grow on oleic acid as well as methanol, we examined them using EM for their ability to induce peroxisomes upon incubation in oleic acid–containing medium for 16–18 h. Of the seven complementation groups examined, *pas1, pas2, pas4, pas5, pas6, pas7,* and *pas8* sections of all except *pas8*, displayed only occasional

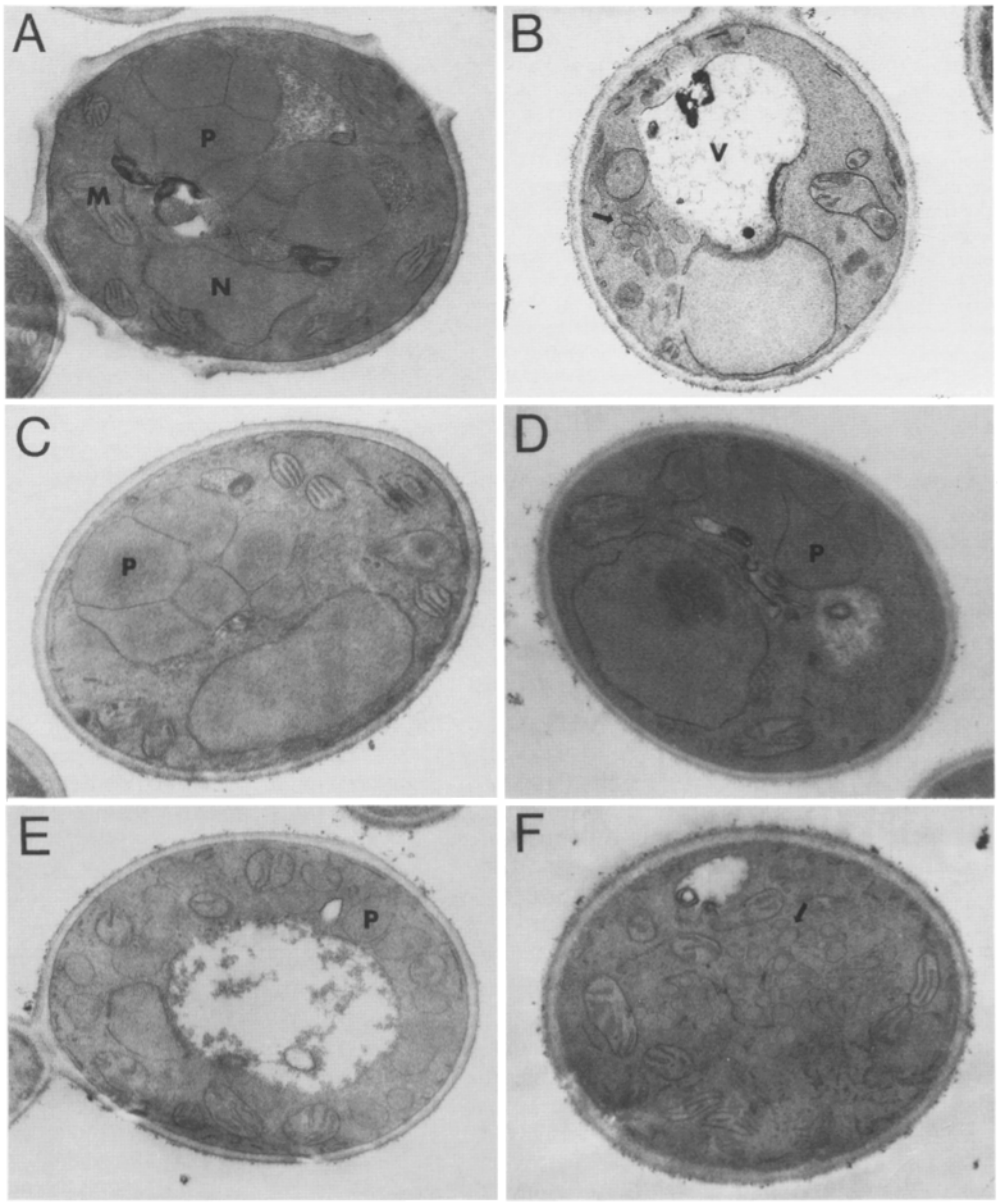

Figure 1. pas8 cells are deficient in growth but not proliferation of peroxisomes. (*A*) Wild-type *P. pastoris* grown in methanol medium. Note the large clustered peroxisomes (*P*); mitochondria (*M*); vacuole (*V*); and nucleus (*N*). Magnification, 20,000. (*B*) The *pas8* mutant induced in methanol medium lacked the large peroxisomes seen in wild-type *P. pastoris*, but did contain clusters of small peroxisome-like structures as indicated by the arrow. (*C*) *pas8* cells containing the complementing plasmid p2-18 regained the ability to form large peroxisomes after growth in methanol medium. (*D*) The *fdh* mutant, like *pas8*, was unable to grow on methanol medium, but, unlike *pas8*, could form large peroxisomes in response to induction by methanol. (*E*) Wild-type *P. pastoris* grown in oleate medium contained numerous peroxisomes (*P*), scattered throughout the cell. (*F*) *pas8* cells induced in oleate medium proliferated large numbers of small peroxisome-like structures, as indicated by the arrow.

small peroxisomal structures, much like their phenotype after incubation in methanol medium (Spong, A. P., D. McCollum, and S. Subramani, unpublished results). In contrast, numerous peroxisome-like profiles were frequently observed in *pas8* cells incubated on oleic acid–containing medium (Fig. 1 *F*, see *arrow*). The peroxisome-like structures of *pas8* cells differed from peroxisomes of wild-type cells in both morphology and number. Generally, sections of wild-type cells grown on oleic acid medium exhibited 2–8 peroxisomes per cell which were round or oval in shape (Fig. 1 *E*). In *pas8* cells, as many as 30 peroxisome-like structures were often observed clustered in one part of the cell. These structures were smaller and more flattened than those of wild-type cells. Indeed they often appeared to be budding. This unusual peroxisome-like morphology is very similar to that seen in cells of the yeast *Candida boidinii* that are undergoing rapid peroxisome proliferation (Veenhuis and Goodman, 1990). The peroxisome-like structures seen in *pas8* cells incubated in oleic acid medium differed from those seen in methanol medium, in that they were much more abundant and appeared larger in size but still smaller than those seen in wild-type cells grown on oleate.

pas8 Cells Are Able to Induce Peroxisomal Proteins on Methanol

Studies in yeast have shown that overexpression of peroxisomal proteins is sufficient to cause growth but not proliferation of peroxisomes (Godecke et al., 1989). In contrast, *pas8* cells incubated in methanol medium are able to proliferate peroxisome-like structures, but not enlarge them. This result suggests that *pas8* cells are either unable to induce peroxisomal proteins in response to methanol or are defective in the import of methanol-induced peroxisomal proteins. To distinguish between these alternatives, we checked whether or not *pas8* cells could induce the three major methanol-induced peroxisomal proteins of *P. pastoris*;

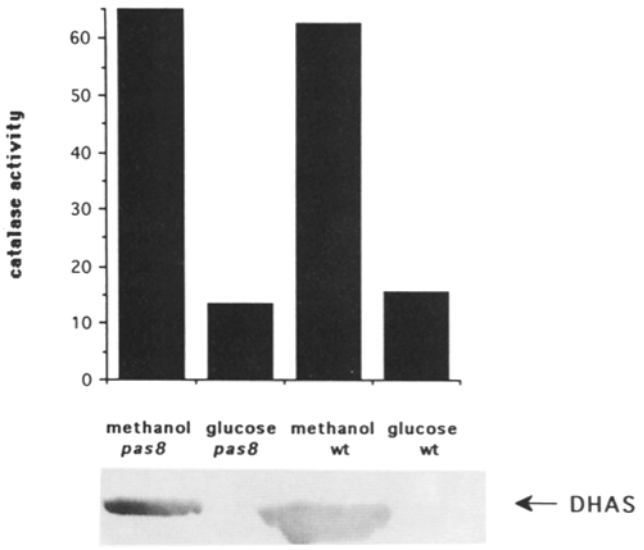

Figure 2. pas8 cells induce peroxisomal proteins in response to methanol. Wild-type and *pas8* cells were precultivated in rich dextrose medium (YPD), and then shifted to either YPD or rich methanol medium (YPM) for 16 h. Whole cell extracts from these cells were assayed for catalase activity and by Western blotting for DHAS protein. Both DHAS and catalase were repressed by dextrose, and induced by methanol, in both *pas8* and wild-type cells indicating that *pas8* cells are not defective in the induction of peroxisomal proteins in response to methanol.

methanol oxidase, catalase, and dihydroxyacetone synthase (DHAS). Enzyme assays and Western blotting revealed that *pas8* cells were capable of inducing both DHAS and catalase (Fig. 2). The situation for methanol oxidase was less clear, since all *pas* mutants examined lacked methanol oxidase activity. However, Western blotting revealed that methanol oxidase protein was induced in *pas8* cells, but was present at much lower levels than in wild-type cells (data not shown). It is known that methanol oxidase must be assembled into octamers after import into peroxisomes for it to be active (Roa and Blobel, 1983), thus *pas* mutants may lack methanol oxidase activity because the protein is not imported and is inactive in the cytosol. Furthermore, the low levels of methanol oxidase found in *pas8* cells are similar to results seen in Zellweger syndrome cells in which certain peroxisomal proteins are unstable and rapidly degraded when mislocalized to the cytosol (Lazarow and Moser, 1989, and references therein). These results indicate that *pas8* cells induce peroxisomal proteins in response to methanol but may be defective in their import into peroxisomes.

pas8 Is Defective in Import of the Three Major Methanol-induced Peroxisomal Proteins

One explanation for the *pas8* phenotype is that *pas8* cells are unable to import peroxisomal proteins induced by incubation on methanol, but they can import some but not all of the proteins induced by incubation on oleic acid–containing medium. To investigate this possibility, we examined the localization of peroxisomal proteins in *pas8* cells. The localization of peroxisomal enzymes in *pas8* cells was analyzed using a differential centrifugation assay to separate cells into an organellar-pellet fraction and a cytosolic supernatant.

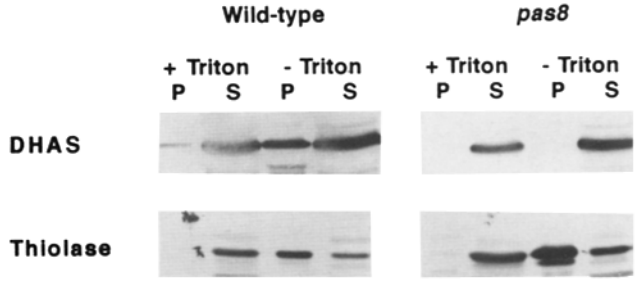

Figure 3. pas8 cells import thiolase but not DHAS into peroxisomes. Wild-type and *pas8* cells were induced 16–18 h in either rich methanol medium (*DHAS* induction) or rich oleate medium (*Thiolase* induction), and then fractionated into cytosolic supernatant and organellar pellet fractions after treatment with and without 0.5% Triton X-100. Wild-type cells displayed pelletable DHAS which was Triton-releasable, indicating that DHAS is imported by wild-type cells. In contrast, *pas8* cells did not appear to import any DHAS into peroxisomes. Thiolase, on the other hand, was imported by both *pas8* and wild-type cells.

First, cells were induced for 16–18 h, and then spheroplasted and broken open by the use of a Dounce homogenizer, followed by two low-speed spins to remove cell debris and nuclei. The remaining supernatant fraction which contains cytosol and organelles was then split into two portions and Triton X-100 (0.5%) was added to one portion in order to rupture organelles. Each fraction was then centrifuged at high speed to pellet the organelles (mainly peroxisomes and mitochondria). The pellet and supernatant fractions were then tested for the presence of peroxisomal proteins either by enzymatic assays or by Western blotting. If a given peroxisomal protein is actually present in the peroxisomes in the pellet fraction then it should get released by Triton X-100 treatment into the supernatant. Growth of *P. pastoris* on methanol requires the three peroxisomal enzymes, methanol oxidase, DHAS, and catalase. *PAS8* (wild-type) cells grown on methanol medium exhibited considerable amounts of DHAS (Fig. 3), as well as enzymatically active methanol oxidase and catalase (Table I) in the organellar pellet, which were in a Triton-releasable form, as expected. In comparison, *pas8* cells did not exhibit any sedimentable catalase (Table I) or DHAS (Fig. 3) indicating the inability of *pas8* cells to import these enzymes into peroxisomes. In contrast to wild-type cells which induce enormous amounts of methanol oxidase upon incubation in methanol medium, pellet and supernatant fractions of *pas8* cells displayed no detectable methanol oxidase activity. Western blotting of *pas8* fractions revealed that methanol-induced *pas8* cells had much reduced levels of methanol oxidase some of which was in a pelletable but not in a Triton-releasable form (data not shown). To localize the methanol oxidase protein in *pas8* cells, we performed immunoelectron microscopy on *pas8* and wild-type cells incubated on methanol using antibodies against the methanol oxidase protein. In wild-type cells, methanol oxidase was assembled into a crystalline core inside the large peroxisomes (Fig. 4, *A* and *B*). However, in *pas8* cells, methanol oxidase was occasionally in small crystalloids located in the cytoplasm (Fig. 5, *A* and *B*) or the immunolabeling revealed that at least 90% of the protein was in diffuse, large, irregular-shaped aggregates in the nucleus (Fig. 5 *C*), or in the cytoplasm (Fig. 5 *D*). <10% of the immunolabeling

Table I. pas8 Cells Are Deficient for Import of Luciferase and Catalase

Cells	Carbon source	Percent of total enzyme activity‡ in organelle pellet					
		Luciferase activity		Catalase activity		Methanol oxidase activity	
		−Triton	+Triton	−Triton	+Triton	−Triton	+Triton
wt	methanol	14.0 (2×10^9)	2.3 (2.2×10^9)	48 (5.13)	6.5 (4.08)	61.0 (5.22)	1.3 (10.69)
pas8	methanol	1.0 (4×10^8)	0.2 (8.3×10^8)	1.1 (5.32)	1.2 (5.66)	ND*	ND
wt	oleate	67.0 (1.1×10^8)	1.7 (1.1×10^8)	83.0 (12.3)	3.2 (9.2)	ND	ND
pas8	oleate	0.8 (3.3×10^7)	0.2 (7.7×10^7)	0.9 (7.46)	1.7 (7.14)	ND	ND

* These extracts did not contain methanol oxidase activity.
‡ The total enzyme activities in the pellet and supernatant fractions are shown in parentheses. The luciferase activity is in arbitrary light units as measured in a luminometer. The catalase activities are in the units as defined in Leighton et al. (1968). The methanol oxidase activity is in μmol of product/min as defined in van der Klei et al. (1990).

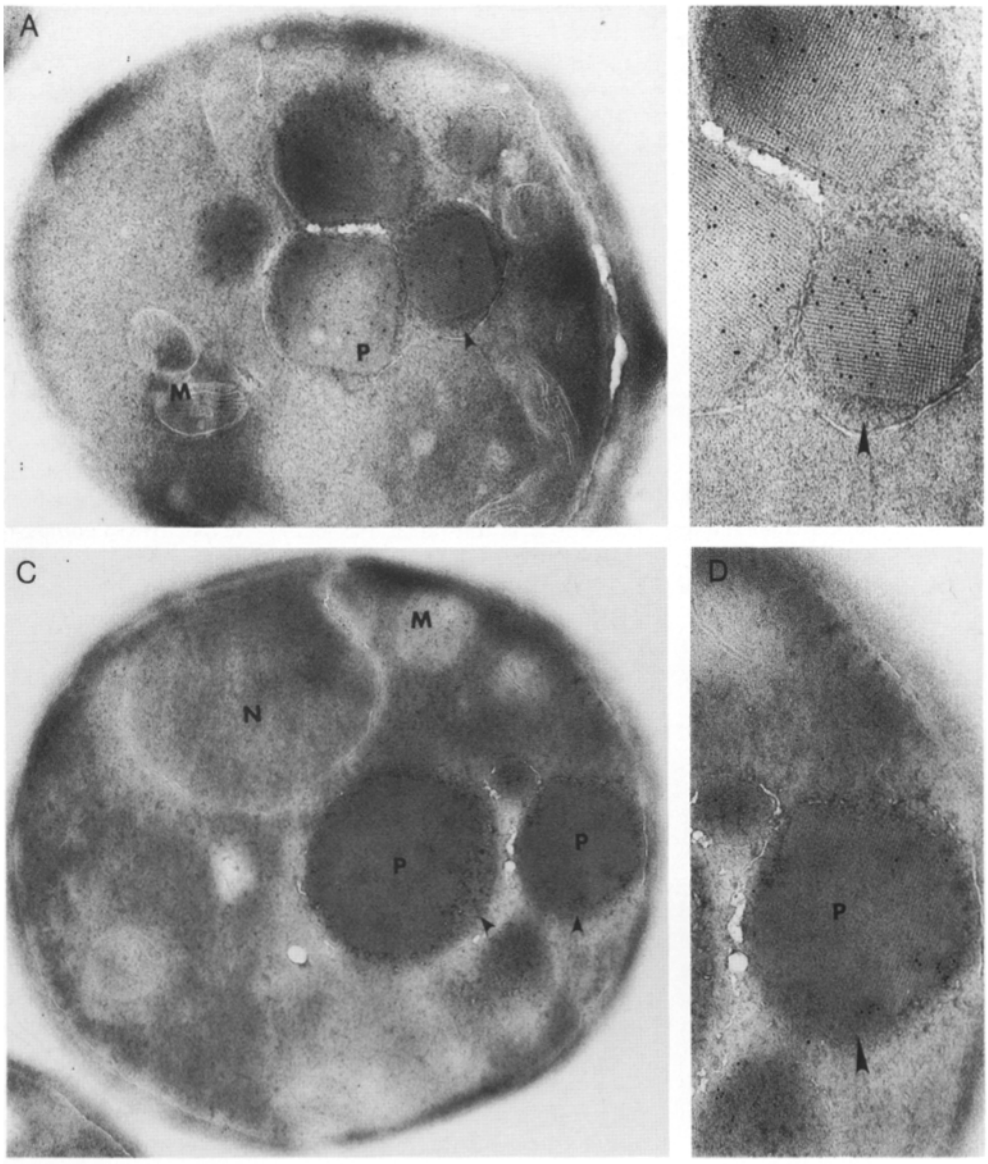

Figure 4. Immunocryoelectron microscopy with anti-methanol oxidase and anti-luciferase antibodies. (*A*) Frozen sections of methanol-grown cells were incubated with anti-methanol oxidase antibodies, followed by incubation with goat anti-rabbit antibodies conjugated to 10-nm gold particles. In wild-type cells, strong labeling for methanol oxidase was observed in the crystalline core of peroxisomes (*P*), which is thought to be composed of methanol oxidase. Other organelles shown include mitochondria (*M*) and nucleus (*N*). Magnification, 33,000. (*B*) Higher magnification of the peroxisomes in *A*. The immunolabeling is over the crystal and is absent in the space between the crystal and the peroxisomal membrane (see *arrow*). Magnification, 66,000. (*C*) Frozen sections of methanol-induced wild-type *P. pastoris* cells, expressing luciferase from the methanol oxidase promoter, were incubated with guinea pig–anti-luciferase antibodies, followed by incubation with mouse anti-guinea pig antibodies conjugated to 5-nm gold particles. Labeling for luciferase was found primarily in the peroxisomes (*P*), indicating that luciferase is imported into peroxisomes in *P. pastoris*. Magnification, 33,000. (*D*) Higher magnification of the immunolabeling in *C* showing labeling in the space between the crystal of methanol oxidase and the peroxisomal membrane. Magnification, 66,000.

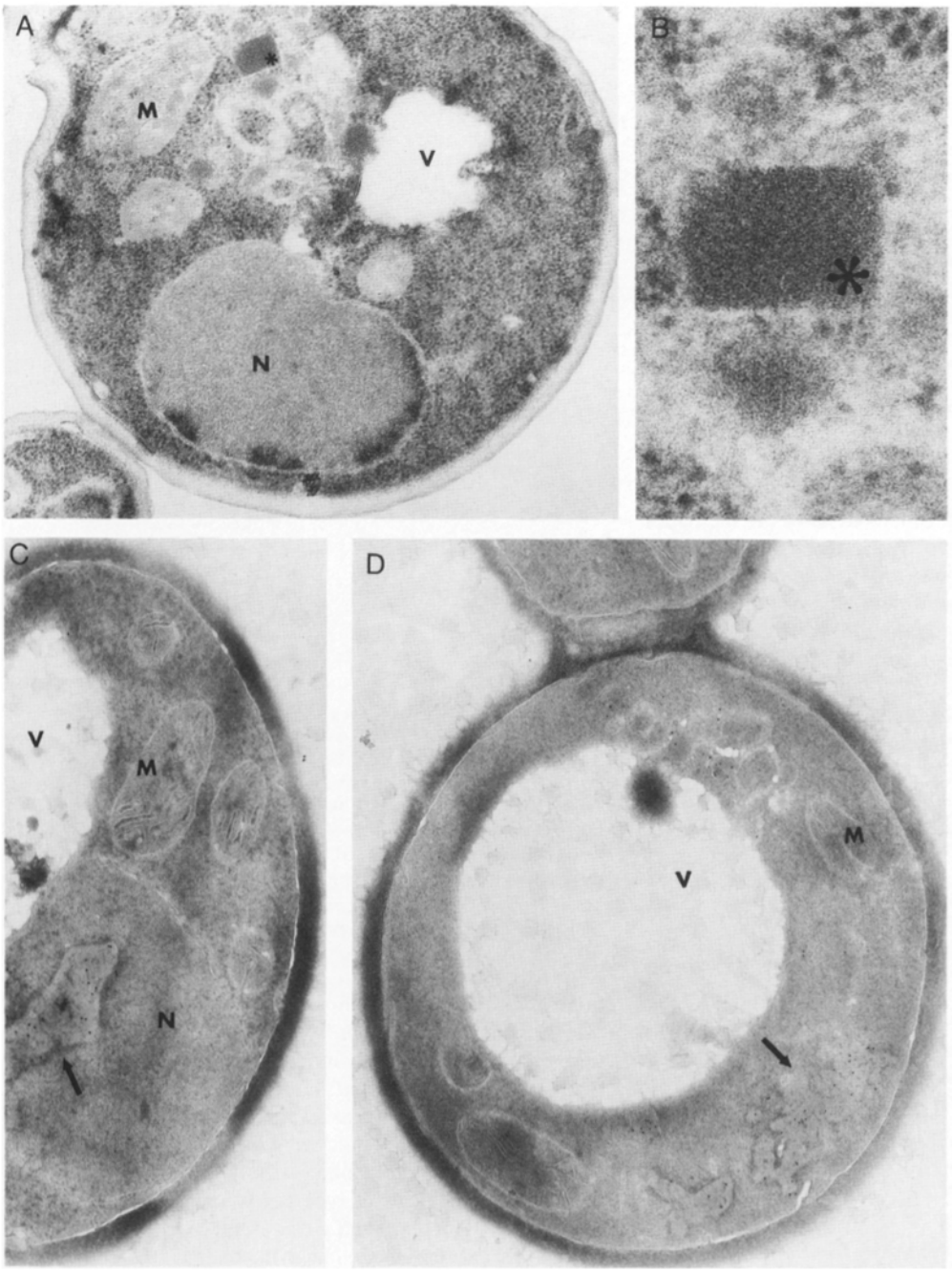

Figure 5. Methanol oxidase is not localized in peroxisomes in *pas8* cells. (*A*) Small cytoplasmic crystalloids of methanol oxidase (see *asterisk*) were observed occasionally in methanol-induced *pas8* cells embedded in Epon resin. Magnification, 33,000. (*B*) Higher magnification of the crystal of methanol oxidase (see *asterisk*) shown in *A* demonstrating the absence of a membrane surrounding the crystal. Magnification, 155,000. (*C*) Immunogold labeling with anti-methanol oxidase antibodies revealed that aggregates of methanol oxidase were occasionally seen in the nucleus (*N*) of *pas8* cells, as indicated by the arrow. Magnification, 33,000. (*D*) Immunogold labeling for methanol oxidase in *pas8* cells was found mainly in large irregular-shaped cytoplasmic aggregates, as indicated by the arrow. Magnification, 33,000.

for methanol oxidase was found in membrane-enclosed structures (see Discussion). Nuclear localization of methanol oxidase has been observed in *pas* mutants of *H. polymorpha* (van der Klei et al., 1991). This unusual localization of methanol oxidase in *pas* mutants could be explained by the presence of a cryptic nuclear localization signal in the protein. These results suggest that like DHAS and catalase, methanol oxidase is not imported into peroxisomes in *pas8* cells but unlike DHAS and catalase, it is unstable in the cytosol, forming insoluble aggregates. Thus, *pas8* cells appear unable to import any of the three main peroxisomal enzymes induced by incubation on methanol medium.

pas8 Cells Import Thiolase

We examined whether or not *pas8* could import any of the peroxisomal enzymes associated with growth on oleic acid medium. Wild-type and *pas8* mutant cells were both induced on oleate medium for ~16 h, and then fractionated into organellar pellet and cytosolic supernatant fractions as described above. In agreement with previous results, *pas8* pellet fractions exhibited no Triton-releasable catalase activity, whereas wild-type cells had 83% of total catalase in a Triton-releasable pellet (Table I). These results indicate that *pas8* cells induced on oleic acid, like those induced on methanol, are unable to import catalase. Western blotting of frac-

tions using thiolase antibody revealed that *pas8* cells import thiolase as do wild-type cells (Fig. 3). This is in contrast to all other *pas* mutants examined which appear unable to import thiolase (Spong, A. P., D. McCollum, and S. Subramani, unpublished observations). To ensure that the thiolase import pathway is intact in methanol grown cells, we expressed the *S. cerevisiae* thiolase from the *P. pastoris* methanol oxidase promoter in *pas8* cells induced in methanol medium. Fractionation of these cells indicated that thiolase is imported in *pas8* cells induced in methanol medium (data not shown). These results indicate that the thiolase import pathway is intact in *pas8* cells induced by oleate or methanol.

pas8 Cells Are Unable to Import Luciferase

Although we suspect that methanol oxidase, DHAS, and catalase use a variant of the carboxy-terminal SKL targeting signal, we wished to confirm that the SKL-dependent import pathway was defective in *pas8* cells. To do this, we expressed the firefly luciferase gene under the control of the methanol oxidase promoter in both wild-type and *pas8* cells. This promoter gives high expression of luciferase on methanol medium, but low expression on oleic acid–containing medium. Luciferase uses the prototypical SKL targeting signal and has been shown to be imported into peroxisomes of diverse eukaryotic species including fungi, plants, and mammals (Gould et al., 1990). Wild-type and *pas8* cells containing the methanol oxidase–luciferase plasmid were induced overnight on either methanol- or oleic acid–containing media, and then analyzed for import of luciferase by the differential centrifugation assay. *pas8* cells were unable to import luciferase after incubation on both oleic acid and methanol. In wild-type cells grown on oleic acid, luciferase was imported quite efficiently with 67% present in the pellet in a Triton-releasable form (Table I). In contrast, luciferase was not imported very efficiently in wild-type cells grown on methanol, with only 14% being present in the pellet in a Triton-releasable form. This result is probably not due solely to breakage of peroxisomes because in the same assay, both catalase and methanol oxidase are pelleted efficiently (48 and 61%, respectively). We suspect that because of the high levels of expression from the methanol oxidase promoter in methanol-grown cells, luciferase cannot be completely imported, although immunoelectron microscopy indicated that luciferase is imported into peroxisomes by wild-type cells grown on methanol (Fig. 4, *C* and *D*). In conclusion, these experiments indicate that *pas8* cells are defective in the import of peroxisomal proteins containing the SKL targeting signal irrespective of the media they are grown on.

Peroxisomes Purified from pas8 Cells Lack Several Proteins

To determine if oleate-induced *pas8* cells could import other proteins besides thiolase, we purified *pas8* peroxisomes and compared their protein profile with that obtained with wild-type peroxisomes on silver-stained polyacrylamide gels (Fig. 6). Peroxisomes from *pas8* cells were purified by first taking oleic acid–induced cells through the differential centrifugation procedure described above. The organellar pellet was then loaded onto a discontinuous Nycodenz step gradient and centrifuged to separate the peroxisomes from other

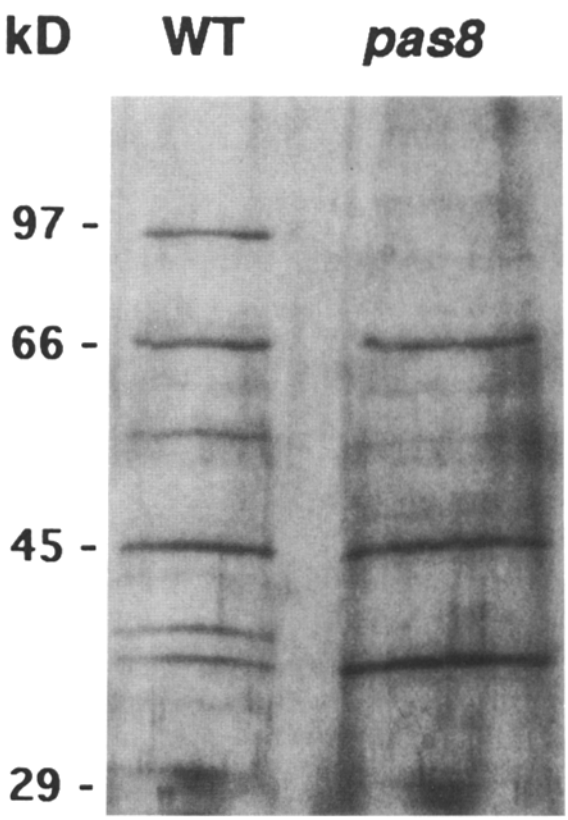

Figure 6. Peroxisomes purified from *pas8* cells lack several proteins. Purified peroxisomes from oleate-induced wild-type and *pas8* cells were run on an SDS-polyacrylamide gel and analyzed by silver staining. Wild-type peroxisomes contained six major bands (MW: 97, 66, 56, 45, 38, and 36 kD), three of which (MW: 66, 45, and 36 kD) were present in *pas8* peroxisomes.

organelles. Fractions from this gradient were analyzed by Western blotting for the presence of thiolase. Thiolase reactivity was found at a density similar to that at which wild-type peroxisomes are found. The thiolase-containing fractions lacked catalase activity as expected, and were essentially free of mitochondria which were at the top of the gradient. A portion of *pas8* peroxisomes were run on a polyacrylamide gel next to purified wild-type peroxisomes (a gift from A. Spong and J. Heyman) and silver stained (Fig. 6). Examination of this gel reveals six major bands (MW: 97, 66, 56, 45, 38, and 36 kD) in wild-type peroxisomes, three of which (MW: 66, 45, and 36 kD) are present in *pas8* peroxisomes. The 97-kD protein present only in wild-type peroxisomes has been found to react with anti-SKL antibodies (data not shown). We suspect that this protein is the trifunctional beta-oxidation enzyme because it is similar in size to the *S. cerevisiae* trifunctional enzyme which ends in SKL (Hiltunen et al., 1992). Of these three common bands, the 45-kD protein has been identified as thiolase but the identities of the other two remain to be determined. It would be interesting to determine the identities of these other two proteins to see if they use a thiolase-type import signal or a third, as yet unidentified, peroxisomal targeting signal. These results show that oleic acid–induced *pas8* cells are unable to import at least two other peroxisomal proteins besides catalase and luciferase.

Molecular Characterization of the PAS8 Gene

As a first step towards a better understanding of *PAS8* function we cloned the *PAS8* gene. First, *pas8 arg4* cells were transformed with a *P. pastoris* genomic library made in the *P. pastoris* replicating vector pSG464 (Gould et al., 1992). After selecting for Arg$^+$ transformants, colonies were pooled and plated onto minimal methanol plates. DNA was isolated from colonies that grew on methanol (Mut$^+$), and transformed into *E. coli*. A single plasmid (designated p2-18) was isolated in this manner that could, upon retransformation into *pas8* cells, complement the *pas8* mutation. Furthermore, *pas8* cells transformed with plasmid p2-18 not only grew on methanol and oleic acid media but regained the ability to import peroxisomal proteins (data not shown) and to form wild-type peroxisomes (Fig. 1 *C*) when grown on minimal methanol medium. This plasmid was found to contain a 5.0-kb insert of which a 2.3-kb HindIII–SpeI fragment was found to fully complement the *pas8* mutation. Sequence analysis of this region revealed a 1.7-kb open reading frame which is predicted to code for a 65-kD protein (Fig. 7). In vitro transcription and translation of this region resulted in a single product that ran slightly larger than predicted at ~68 kD (data not shown).

Initial database searches revealed no striking homologies between *pas8* and other proteins. But more careful analysis showed that the carboxy-terminal half of *PAS8* exhibited slight but extended homologies to several other genes: the *Schizosaccharomyces pombe Nuc2* gene, the *S. cerevisiae CDC23, SSN6,* and *CDC16* genes, and the *Aspergillus nidulans BimA* gene. The regions of identity with these genes were confined to the tetratricopeptide repeat (TPR) motif that all of these genes possess (Fig. 8 *B*). The TPR motif consists of a highly degenerate 34-amino acid repeat initially identified in the *CDC23* gene (Sikorski et al., 1990). TPR motifs have been identified in genes involved in diverse biological processes including nuclear division (*nuc2*$^+$, *bimA*, *CDC23,* and *CDC16*), RNA processing (*PRP6* of *S. cerevisiae*), and mitochondrial protein import (*MAS70* of *S. cerevisiae*). At present nothing is known about the function of the TPR domains in these genes, although the TPR motifs have been shown to be essential for function in *Nuc2, CDC23,* and *SSN6.* In fact temperature-sensitive mutations in *Nuc2* and *CDC23* have been shown to map to conserved residues in the TPR domain (for review see Goebl and Yanagida, 1991; Sikorski et al., 1991). *PAS8* contains seven consecutive TPR motifs in the carboxy-terminal half of the protein followed by a 52-amino acid tail region. Fig. 8 *A* shows the alignment of the *PAS8* TPR motifs with only consensus residues outlined, although inspection reveals considerable conservation of similar amino acids outside of the consensus. The amino-terminal half of the protein is extremely glutamine rich containing 20% glutamine residues, but displays no significant homology to other proteins. Also, because the PAS8 protein does not possess transmembrane domains it seems likely that PAS8 is not a peroxisomal integral membrane protein.

Cells with a Disruption of the PAS8 Gene Have the Same Phenotype as pas8.1 Cells

We used homologous recombination to replace most of the *PAS8* gene, including the conserved TPR repeats with the *P. pastoris ARG4* gene. Proper targeting of this construct to the

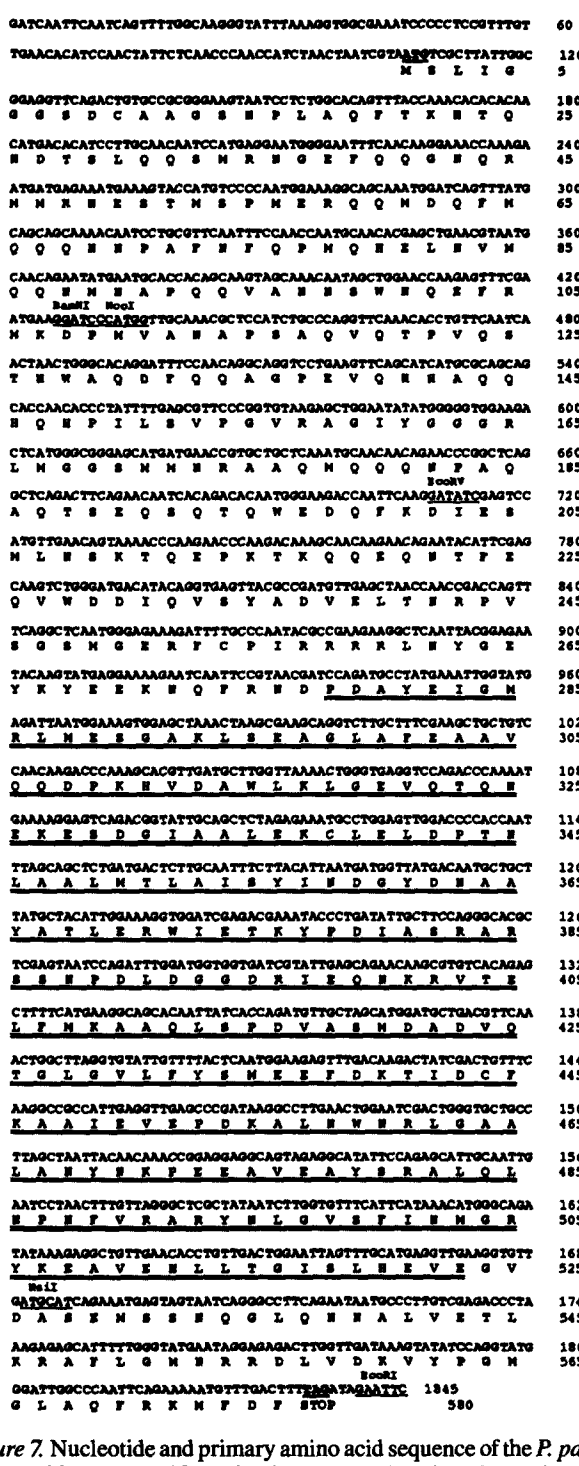

Figure 7. Nucleotide and primary amino acid sequence of the *P. pastoris PAS8* gene. *PAS8* nucleotide and deduced amino acid sequence. The translation of the *PAS8* sequence from the ATG, at nucleotide 106 (bold, underlined) to the termination codon (*STOP*) at nucleotide 1834, is shown below the first nucleotide of each codon in the sequence. The amino acids of the protein are in the one-letter code. The TPR domain of *PAS8* is double underlined. Relevant restriction sites are indicated. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z19592.

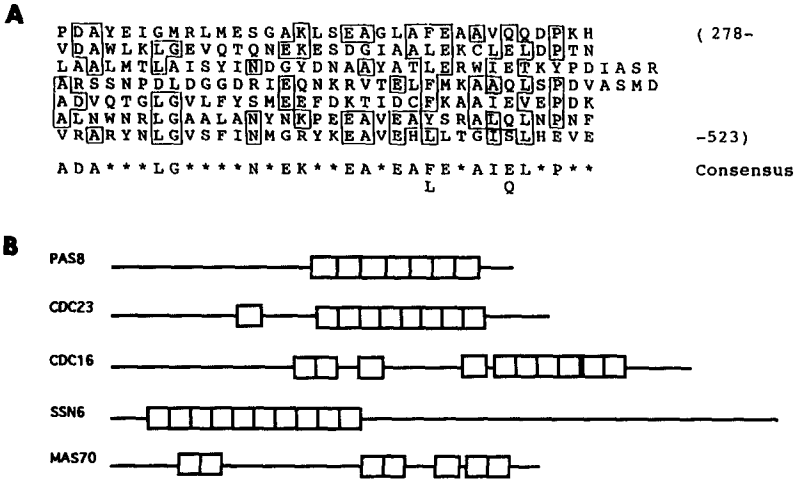

Figure 8. PAS8 is a member of the TPR protein family. (*A*) Alignment of the seven consecutive TPR motifs (amino acids 278–523) of the PAS8 sequence. If an amino acid was present at a given position in 3 out of 7 repeats it was termed a consensus residue. Consensus residues are outlined in the repeats, and given in the consensus sequence written below the repeats. (*B*) Schematic diagram showing the location of the TPR motifs as boxes in PAS8 and several other TPR-containing proteins.

chromosome was confirmed by Southern blotting (Fig. 9). Disruption of the *PAS8* gene resulted in viable cells that were unable to form normal peroxisomes after methanol induction (data not shown) or to grow on methanol- or oleic acid–containing media. This strain was also unable to complement the *pas8.1* mutation showing that the Δ*pas8* mutation was in the same complementation group as the original *pas8.1* mutation. Furthermore, the Δ*pas8* cells retained the ability to import thiolase (Fig. 10 *B*), but not catalase (Fig. 10 *A*), suggesting that the original *pas8.1* mutation represents a null allele.

PAS8 Protein Localizes to Peroxisomal Membranes and Is Induced by Methanol and Oleic Acid

Overexpression of PAS8 protein in *E. coli* was achieved by fusing a portion of the *PAS8* gene to the bacterial *LacZ* gene. This fusion protein was injected into rabbits to generate polyclonal antibodies. Western blotting with PAS8 antibodies on *P. pastoris* whole cell extracts identified a single band of ~68 kD, which was present in glucose-grown cells, but was induced severalfold by growth on methanol and oleic acid media (Fig. 11 *A*). Interestingly, when organellar pellet and cytosolic supernatant fractions of wild-type cells induced on methanol or oleic acid media were examined, PAS8 was found primarily in the organellar pellet (data not shown). This organellar pellet, which consists mainly of peroxisomes and mitochondria, was loaded onto a sucrose density gradient to separate peroxisomes from mitochondria. Peak mitochondrial and peroxisomal fractions were determined by assaying for the marker enzymes catalase (peroxisomes) and succinate dehydrogenase (mitochondria). Western blotting of equivalent amounts of protein from each of these fractions revealed that PAS8 protein is found almost exclusively in peroxisomes. The small amount of PAS8 in the mitochondrial fraction reflects the peroxisomal contamination found in this fraction as shown by the presence of a low level of catalase activity in this fraction (Fig. 11 *B*).

To gain information about the intra-peroxisomal localization of PAS8, organelle-pellet fractions from methanol-induced wild-type cells were subjected to pH 8.5, and low osmolarity, and separated into membrane-pellet (Fig. 11 *C*, lane *3*) and supernatant fractions (Fig. 11 *C*, lane *2*). These conditions have been shown to cause peroxisomes to rupture

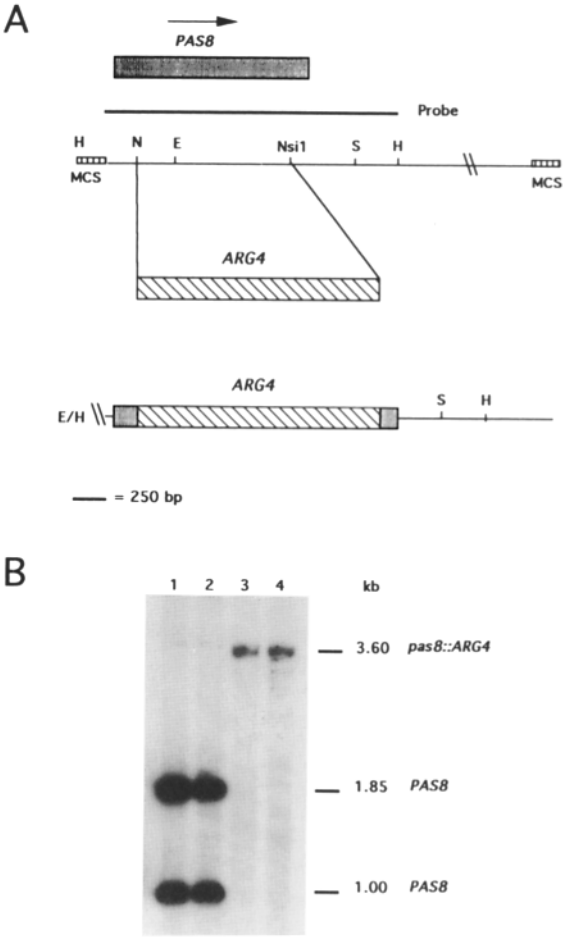

Figure 9. Disruption of the *PAS8* gene. (*A*) The *PAS8* coding region contained between the NcoI (*N*) and NsiI sites was replaced with the *P. pastoris ARG4* gene and introduced into the genome by homologous recombination. The unique SpeI site of p2–18 is also shown. (*B*) Proper targeting of this construct to the genome was confirmed by Southern blotting of genomic DNA cut with EcoRV (*E*) and HindIII (*H*), and probed with the indicated probe. (lanes *1* and *2*) DNA from wild-type *P. pastoris*; (lanes *3* and *4*) DNA from two independent Δ*pas8* strains.

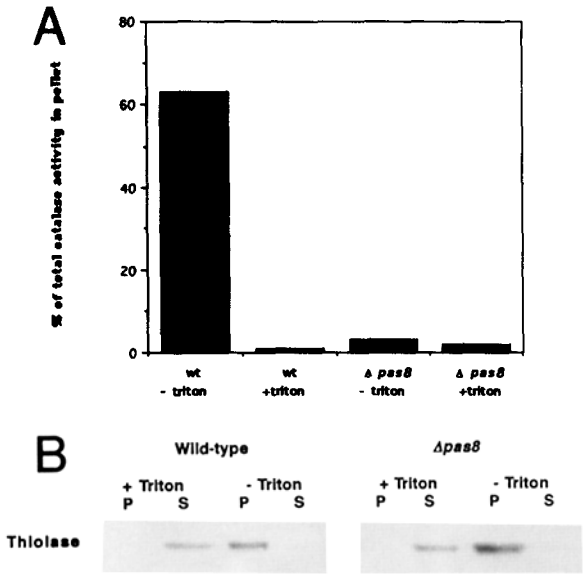

Figure 10. Δ*pas8* cells import thiolase but not catalase. Fractionation of Δ*pas8* cells induced in oleate medium showed they did not import catalase (*A*), but did import thiolase (*B*).

and release matrix proteins (Nuttley et al., 1990). Under these conditions, PAS8 protein was found exclusively in the membrane-pellet fraction. In a second experiment, organelle-pellet fractions were treated with both pH 8.5 and 1 M NaCl, and separated into membrane-pellet (Fig. 11 *C*, lane *5*) and supernatant fractions (Fig. 11 *C*, lane *4*), and again, PAS8 was found mainly in the membrane pellet. These experiments indicate that PAS8 is tightly associated with the peroxisomal membrane.

Western blotting revealed that PAS8 protein was not present in pellet or supernatant fractions from *pas8.1* cells induced on methanol or oleic acid, but the introduction of complementing plasmid p2–18 into *pas8.1* cells resulted in the appearance of PAS8 in the organellar pellet (data not shown).

In Vitro Translated PAS8 Protein Binds to the SKL Peroxisomal Targeting Signal

Given that *pas8* was the only mutant isolated in our screen that was deficient solely for import of PTS1-, but not PTS2-containing proteins, we wished to determine if PAS8 protein could bind to the PTS1 targeting signal. To do this we used PAS8 protein that had been translated in vitro in a rabbit reticulocyte lysate, and assayed whether or not it could bind to a 12–amino acid peptide, ending in the amino acids SKL, coupled to HSA, which was in turn coupled to agarose

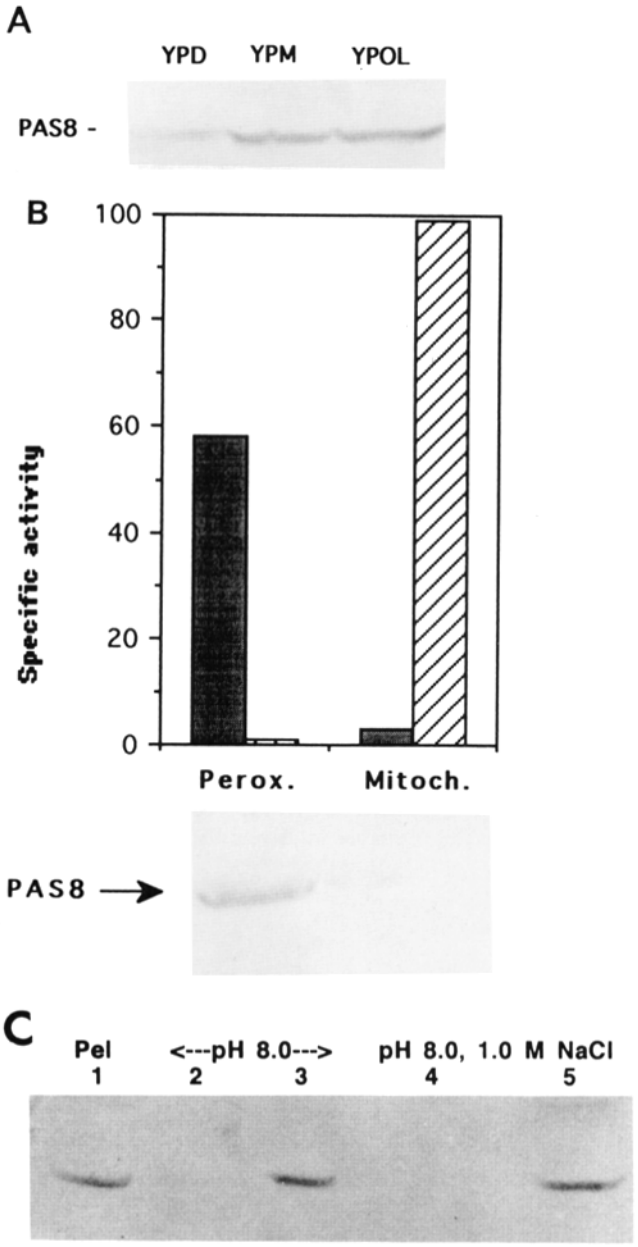

Figure 11. Identification of the PAS8 protein in *P. pastoris*. (*A*) Whole cell extracts of wild-type cells grown with dextrose, methanol, or oleate as carbon source were probed by Western blotting with a 1:150 dilution of anti-PAS8 antiserum. PAS8 protein was induced severalfold by methanol and oleate from its level in dextrose-grown cells. (*B*) Peak peroxisomal and mitochondrial fractions were assayed for the peroxisomal marker enzyme catalase and the mitochondrial marker enzyme succinate dehydrogenase. The bar graph shows catalase specific activity (*solid bar*) in B.U./ml per μg protein (Leighton et al., 1968), and succinate dehydrogenase specific activity (*crosshatched bar*) expressed as ΔA550/min per μg of protein (ΔA550 is the change in absorbance at 550 nm, Sottocasa et al., 1967) in each fraction. The graph shows that there was only minimal cross-contamination between the two fractions. 40 μg of protein from each fraction were probed by Western blotting with antiserum against PAS8 protein. PAS8 was found almost exclusively in the peroxisomal fraction, indicating that PAS8 is a peroxisomal protein. (*C*) An organelle-pellet fraction from wild-type cells induced in methanol medium for 16–18 h (lane *1*). A portion of this pellet was suspended in Tris buffer at pH 8.5 (see Materials and Methods) to rupture peroxisomes and centrifuged at 100,000 *g*. Equivalent portions of the resulting supernatant (lane *2*) and pellet (lane *3*) were loaded. Similarly, a portion of the organelle pellet was suspended in Tris buffer containing 1 M NaCl at pH 8.5 (see Materials and Methods) and centrifuged at 100,000 *g*. Equivalent portions of the resulting supernatant (lane *4*) and pellet (lane *5*) fractions were loaded. All fractions were analyzed by Western blotting with anti-PAS8 antibodies.

beads. Labeled in vitro translated PAS8 protein was incubated with HSA-SKL beads, or HSA alone coupled to beads, for 1 h at room temperature. The supernatant containing unbound PAS8 protein was then removed, and the beads were washed thoroughly to remove nonspecifically bound material. Sample buffer was added to supernatant and bead-containing pellet fractions, and equal portions of each fraction were run on SDS-polyacrylamide gels for fluorography. PAS8 protein was found to bind to HSA-SKL beads (Fig. 12, lanes *3* and *4*), but not to HSA alone coupled to beads (Fig. 12, lanes *1* and *2*). Next, we assayed whether or not PAS8 would bind to a 9-amino acid peptide identical to the SKL peptide except that it lacked the last three amino acids SKL (ΔSKL). This ΔSKL peptide was coupled to HSA (HSA-ΔSKL), and in turn coupled to beads, and then assayed for binding. PAS8 protein would not bind to the HSA-ΔSKL beads, indicating that the binding was specific for the last three amino acids SKL (Fig. 12, lanes *7* and *8*). Finally it was shown that PAS8 would bind to beads to which just the SKL peptide was coupled (Fig. 12, lanes *5* and *6*). Preliminary experiments suggest that PAS8 binds directly to the SKL targeting signal. Reticulocyte lysate was first incubated with HSA-SKL beads to deplete it of endogenous SKL-binding factors under conditions where the lysate was unable to support cytosol-dependent peroxisomal protein import in permeabilized CHO cells (Wendland and Subramani, 1993). The PAS8 protein translated in this depleted lysate was still able to bind to HSA-SKL, but not HSA (data not shown) indicating that PAS8 probably binds the SKL targeting signal directly.

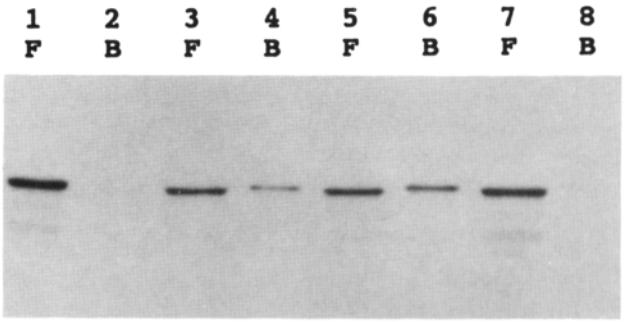

Figure 12. PAS8 protein binds the SKL peroxisomal targeting signal. Labeled in vitro translated PAS8 protein was assayed for binding to HSA coupled beads. (Lanes *1* and *2*) Fractions containing free (*F*) and bound (*B*) PAS8, respectively. (lanes *3* and *4*) Binding to beads coupled with HSA conjugated to a peptide ending in the PTS1 signal SKL. (lanes *5* and *6*) PAS8 binding to beads coupled with the SKL peptide alone. (lanes *7* and *8*) PAS8 binding to beads coupled with HSA conjugated to the ΔSKL peptide (see text).

Discussion

The pas8 Mutant Is Deficient in the Import of Peroxisomal Matrix Proteins Containing the PTS1

In this study, we report the characterization of the *pas8* mutation as well as the cloning and sequence of the *PAS8* gene. Our data show that *pas8* cells are not deficient either in peroxisome proliferation or segregation to daughter cells. The inability of *pas8* cells to import luciferase suggests that the *PAS8* gene is essential for the import of peroxisomal proteins bearing PTS1-type (SKL) targeting signals. Notably, this import deficiency was independent of the carbon source that the cells were grown on. The finding that the 97-kD protein, found in peroxisomes of wild-type but not of *pas8* cells, reacts with anti-SKL antibodies further supports this notion (Spong, A. P., and S. Subramani, unpublished observations). The *P. pastoris* methanol oxidase protein ends in the sequence ARF (Koutz et al., 1989) which is quite similar to the carboxy terminus of the *S. cerevisiae* (Cohen et al., 1988) and the fruitfly (Orr et al., 1990) peroxisomal catalase which end in SKF. The last six amino acids of the *S. cerevisiae* catalase gene are sufficient for peroxisomal targeting in yeast (Kragler et al., 1993), and the sequence ARF is known to direct peroxisomal targeting of heterologous proteins (Roggenkamp, 1992). Furthermore, the finding that the sequence AKI can act as a peroxisomal targeting signal for the yeasts *Candida tropicalis* and *S. cerevisiae* shows that greater degeneracy of the COOH-terminal residue may be allowed for PTS1-type targeting signals in yeast (Aitchison et al., 1991). Finally, the DHAS gene of *P. pastoris* ends in the sequence DKL (Tom Vedvick, personal communication), which is also similar to the PTS1 targeting signal. These observations suggest that methanol oxidase, catalase, and DHAS may use PTS1-type peroxisomal targeting signals explaining their mislocalization in *pas8* cells. We cannot exclude the possibility that <10% of the alcohol oxidase might be in membrane-bound vesicles. Whether this represents engulfment of cytoplasmic aggregates of the protein by vacuoles or transport of a small amount of alcohol oxidase to peroxisomes by the use of redundant signals, such as those found by Kragler et al. (1993), is not clear at present.

pas8 Cells Do Import Proteins with PTS2 into Peroxisomes

In contrast, *pas8* cells import thiolase, independent of the carbon source that the cells are grown on. Molecular and genetic evidence indicates that thiolase is imported by a pathway that is at least partially independent of the PTS1 import pathway. Molecular analysis has shown that the rat thiolase uses an amino-terminal signal (PTS2) that has no sequence similarity to the PTS1 signal (Swinkels et al., 1991; Osumi et al., 1991). Genetic analysis in *S. cerevisiae* has identified a mutant, *pas7*, that is defective in the import of thiolase but not other peroxisomal proteins including one ending in SKL (Kunau and Hartig, 1992).

Conversely, some Zellweger complementation groups do import thiolase but not other peroxisomal proteins into "peroxisomal ghosts" (Balfe et al., 1990). Experiments using Zellweger syndrome fibroblasts indicate that some complementation groups are unable to import peroxisomal proteins bearing the PTS1 signal (Walton et al., 1992). Thus the import defect in *pas8* cells mimics that found in Zellweger syndrome cells. It is also worth noting that *pas8* cells exhibit peroxisome ghosts on methanol and oleate (Fig. 1, *B* and *F*) as do Zellweger syndrome cells.

Peroxisome Proliferation and Enlargement Are Distinct Steps in Peroxisome Biogenesis

Work by a number of labs has suggested that overexpression and import of peroxisomal proteins can cause growth, but not proliferation of peroxisomes (Godecke et al., 1989;

Veenhuis and Goodman, 1990). The phenotype of the *pas8* mutant provides evidence that peroxisome proliferation and growth are two distinct steps in peroxisome biogenesis that can be uncoupled genetically. Furthermore, it is clear that many of the peroxisomal matrix proteins induced in *pas8* cells are never imported into the peroxisomes. This observation, combined with the results of Veenhuis and Goodman (1990) which indicate that in *C. boidinii* peroxisome proliferation precedes temporally the induction and import of peroxisomal matrix enzymes, strongly suggests that the induction of peroxisomal matrix proteins may drive the enlargement of peroxisomes, but not their proliferation. This model of the involvement of distinct cues for peroxisome proliferation and enlargement would explain why the overexpression of DHAS in glucose-grown *S. cerevisiae* induces peroxisome enlargement but not proliferation (Godecke et al., 1989).

It should also be noted that although peroxisome proliferation and enlargement are defined as temporally and genetically separable events, neither is obligatory for the other to happen. Peroxisome growth has been observed in *S. cerevisiae* in the absence of organelle proliferation (Godecke et al., 1989), and the phenotype of the *pas8* mutant illustrates peroxisome proliferation in the absence of growth.

Functions of the PAS8 Protein

The cloning of the *PAS8* gene and identification of its corresponding gene product should allow for a greater understanding of the function of PAS8 in peroxisomal protein import. PAS8 has been found to contain seven imperfect 34–amino acid repeating motifs known as TPR motifs. Although these motifs have been found in a variety of genes involved in diverse cellular pathways, virtually nothing is known about their true function. It has been speculated that TPR motifs may be involved in membrane association through amphipathic helices or perhaps protein–protein interactions (Sikorski et al., 1990). Modeling studies suggest that TPR motifs form interlocking alpha helices (Hirano et al., 1990). It has also been suggested that many TPR genes appear to interact genetically with proteins containing a 43–amino acid repeat called the beta-transducin repeat. These include *PRP6/PRP4, SSN6/TUP1*, and *CDC16,CDC23/CDC20* (Goebl and Yanagida, 1991, and references therein). In fact the *SSN6* and *TUP1* gene products physically interact, although it is unclear whether this interaction is mediated through their respective repeat motifs (Williams et al., 1991). It will be interesting to see, as more *PAS* genes are cloned and sequenced, whether any of them contain beta-transducin type repeats.

Several lines of evidence suggest that the PAS8 protein is peroxisomal and membrane associated. The enrichment of PAS8 in the organelle pellet and purified peroxisomal fractions (Fig. 11 *B*), shows that it is peroxisomal. The data in Fig. 11 *C* show that it is tightly membrane associated. Furthermore, the PAS8 protein is found in peroxisome ghosts purified from *pas5* mutant cells which lack matrix proteins and are deficient in the transport of both PTS1- and PTS2-containing proteins (Spong, A., and S. Subramani, manuscript in preparation). Finally, the ability of the PAS8 protein to bind the HSA-SKL conjugate and the SKL peptide specifically provides strong support for the possibility that PAS8 is the long-sought-after PTS1 receptor. It is also interesting to note that the PAS8 protein is weakly homologous to the MAS70 protein from *S. cerevisiae*, which is known to be a receptor involved in the import of some but not all mitochondrial proteins (Hines et al., 1990). We do not know the topology of PAS8 or whether it is an integral or peripheral membrane protein. However, based on the absence of an obvious hydrophobic, membrane-spanning domain in PAS8, the presence of the protein in the peroxisome ghosts of *pas5* mutant cells, the lack of a known PTS in PAS8, and its ability to bind the SKL peptide, we hypothesize that PAS8 is a cytoplasmically oriented peripheral membrane protein. However, further experiments will be required to prove this point.

It is interesting that, with the exception of *P. pastoris pas8* and *S. cerevisiae pas7*, and *pas10*, all other *pas* mutants isolated thus far in yeasts appear to be deficient in the import of all peroxisomal matrix proteins tested. The existence of two distinct pathways for the import of peroxisomal matrix proteins can be accommodated in two models which differ in whether one or two distinct translocation machineries are involved in the import of proteins containing PTS1 and PTS2. In the first model, the PTS1 and PTS2 import pathways use different receptors, and then merge to use a common translocation machinery. If this were true then some or all of the mutants (e.g., *pas1–7* of *P. pastoris*) that are affected in the import of all peroxisomal matrix proteins could be true import mutants affecting different components of the translocation machinery. The second model is one in which distinct translocation machineries are involved for PTS1 and PTS2. According to this model, the other *pas* mutants that fail to import all matrix proteins may be deficient in peroxisome proliferation or in general peroxisomal functions unrelated to import, such that the mislocalization of peroxisomal proteins is a secondary consequence of these defects. The cloning and characterization of other genes involved in peroxisome assembly and import should reveal which of these models is true.

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Note Added in Proof. While this work was in progress, Dr. Henk Tabak and his colleagues (University of Amsterdam) cloned and characterized the gene (*PAS10*) that complements the *pas10* mutant of *S. cerevisiae* which exhibits a similar phenotype as the *pas8* mutant of *P. pastoris* (Tabak, H., personal communication). Comparison of the *PAS10* gene of *S. cerevisiae* and the *PAS8* gene of *P. pastoris* shows that they are homologous.

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