Role of the PAS1 Gene of *Pichia pastoris* in Peroxisome Biogenesis

John A. Heyman, Edward Monosov, and Suresh Subramani

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

Abstract. Several groups have reported the cloning and sequencing of genes involved in the biogenesis of yeast peroxisomes. Yeast strains bearing mutations in these genes are unable to grow on carbon sources whose metabolism requires peroxisomes, and these strains lack morphologically normal peroxisomes. We report the cloning of *Pichia pastoris* PAS1, the homologue (based on a high level of protein sequence similarity) of the *Saccharomyces cerevisiae* PAS1. We also describe the creation and characterization of *P. pastoris* pasl strains. Electron microscopy on the *P. pastoris* pasl cells revealed that they lack morphologically normal peroxisomes, and instead contain membrane-bound structures that appear to be small, mutant peroxisomes, or "peroxisome ghosts." These "ghosts" proliferated in response to induction on peroxisome-requiring carbon sources (oleic acid and methanol), and they were distributed to daughter cells. Biochemical analysis of cell lysates revealed that peroxisomal proteins are induced normally in *pasl* cells. Peroxisome ghosts from *pasl* cells were purified on sucrose gradients, and biochemical analysis showed that these ghosts, while lacking several peroxisomal proteins, did import varying amounts of several other peroxisomal proteins. The existence of detectable peroxisome ghosts in *P. pastoris* pasl cells, and their ability to import some proteins, stands in contrast with the results reported by Erdmann et al. (1991) for the *S. cerevisiae* pasl mutant, in which they were unable to detect peroxisome-like structures. We discuss the role of PAS1 in peroxisome biogenesis in light of the new information regarding peroxisome ghosts in *pasl* cells.

Peroxisomes are single membrane-bound organelles ~0.2-1.0 microns in diameter. They are found in virtually all eukaryotic cells and, depending on cell type and growth conditions, vary in abundance from two to several hundred per cell (Lazarow and Fujiki, 1985). The importance of peroxisomes in humans is demonstrated by the class of peroxisomal disorders of which Zellweger syndrome is an example. Affected individuals usually die within several years of birth, and cells from affected individuals have been shown to have non-functional peroxisomes (for review see Lazarow and Moser, 1989).

While the biochemistry of peroxisomes has been well-characterized (Van den Bosch et al., 1992), less is known about peroxisome biogenesis (Subramani, 1993). Peroxisomes are thought to arise from preexisting peroxisomes, just as mitochondria are believed to be derived from preexisting mitochondria (Borst, 1989). This is supported by electron micrographs showing budding peroxisomes (Osumi et al., 1975; Veenhuis et al., 1978). Additionally, peroxisomes contain no protein synthesis machinery, and all peroxisomal matrix and membrane proteins are synthesized on free poly-somes and then imported into the organelle (Subramani, 1993). Given these constraints, cells must do several things to produce and propagate functional peroxisomes: (a) peroxisomal proteins must be synthesized and imported into the organelle; (b) the correct substrates and cofactors must be imported into the organelle; (c) membrane material consisting of lipids and proteins must be transported to peroxisomes so that they can grow and divide; and (d) peroxisomes must be segregated to daughter cells during cell division.

To identify and characterize the proteins involved in these processes, mutants with generalized peroxisomal defects have been generated in several organisms. This approach has led to the cloning and sequencing of several genes essential for peroxisome biogenesis (see Subramani, 1993). Of these genes, only *P. pastoris* PAS8 (McCormick et al., 1993) and the homologous *S. cerevisiae* PAS10 (van der Leij et al., 1993) have been assigned a function that is clearly related to peroxisome biogenesis. In *pas8* *P. pastoris* cells and in *pas10* *S. cerevisiae* cells, proteins normally targeted to the peroxisome by a COOH-terminal PTS1 (peroxisome-targeting signal 1); a COOH-terminal -SKL tri-peptide or variant that has been shown to direct proteins to peroxisomes in

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1. Abbreviations used in this paper: DHAS, dihydroxyacetone synthase; PTS, peroxisome-targeting signal; SDH, succinate dehydrogenase; SC, synthetic medium/citrate; SD, dextrose; SE, ethanol; SG, glycerol; SL, lactate; SM, methanol; SOLT, oleic acid/Tween 40; YPD, yeast extract with peptone; YPD, dextrose; YPM, methanol; YPOLT, oleic acid/Tween 40.
Materials and Methods

Bacterial Strains

The Escherichia coli strain DH5α was the most frequently used strain. Miniprep DNA samples were prepared using a modified alkaline lysis procedure (Zhou et al., 1990). Other recombinant DNA techniques were performed essentially as described previously (Sambrook et al., 1989).

Yeast Strains

The P. pastoris wild-type strain (21-1), the arginine-requiring (GS190) and histidine-requiring (GS115) strains (accession numbers Y14300, Y18014, and Y15851, respectively) were obtained from the Northern Regional Research Laboratories (Peeoria, IL). We refer to these strains as PYY1, PYY3, and PYY4, respectively. PYY12 (arg4 his4) was generated previously by our group (Gould et al., 1992). The PYY300 (arg4 his4 pas8::ScARG4) and the PYY301 (arg4 his4 pas8::ARG4) strains were developed for this study as described below. P. pastoris PYY20 (arg4 pas8::ScARG4) and the PYY20 (arg4 his4 pas8::ARG4) were isolated by Dan McCollum (Gould et al., 1992). PYY115 (arg4 his4 pas8::ARG4) (McCollum et al., 1993) is referred to as Δpas8 in this paper.

Yeast Culture

Standard rich medium for growth of P. pastoris was YPD medium (1% yeast extract, 2% bactopentone, 2% dextrose). Defined synthetic medium consisted of yeast nitrogen base at 0.67% wt/vol supplemented with carbon sources to a final concentration of either 2% dextrose (SD), 0.5% methanol (SM), 1% citrate (SC), 1% lactate (SL), 1% glycerol (SG), or 0.2% oleic acid/0.02% Tween 40 (SOLT). For auxotrophic strains requiring arginine or histidine or both, the required amino acids were included at 40 μg/ml. Culturing, mating, sporulation, and random spore analysis were done as previously described (Gould et al., 1992).

Electron Microscopy

Electron microscopy was performed as described previously (Gould et al., 1992). However, after preculture of cells in defined dextrose medium (SD), cells were induced for 4 h in either SM or SOLT media, except for the cells shown in Fig. 6 a, which were cultured for 7 h in YPM (1% yeast, 2% bactopeptone, 0.5% methanol) after preculture in YPD. These conditions are sufficient for induction of peroxisomes and peroxisomal proteins.

Preparation of Crude Yeast Cell Lysates

Cells were grown exponentially in SD and then switched to SM or SOLT for the desired length of peroxisome induction. Crude cell lysates were prepared by glass-bead lysis. This was performed essentially as previously described (Sambrook et al., 1989) except that the lysate buffer was 62.5 mM Tris-Cl (pH 8.7), 5 mM EDTA, 2 mM PMSF, and lysates were not cleared of debris before use in experiments.

Sucrose Gradient Purification of P. pastoris Organelles

Fractionation of P. pastoris cells into an organellar pellet and supernatant was performed as described previously (Gould et al., 1992), but with the following changes. Cells were grown in a semi-rich inducing media (1% bactopentone wt/vol, 2% wt/vol yeast extract) containing oleic acid and Tween 40 (0.2% vol/vol) of a 9:1 mix of oleic acid and Tween 40, respectively, YPOLT) before being fractionated. Peroxisomes were purified from this organellar pellet on a sucrose gradient as previously described (Nuttley et al., 1990).

Sucrose gradients were drained with the aid of a peristaltic pump and fractions were assayed for catalase and succinate dehydrogenase (SDH) activities as described previously (Sottocasa et al., 1967; Leighton et al., 1968).

In Figs. 9 and 10, the following steps were taken so that the relative band intensities seen for a particular protein in Fig. 9 vs. Fig. 10 would reflect the relative amounts of that protein in the wild-type (Fig. 9) and the Δpas8 mutant (Fig. 10) peroxisomes: (a) 8 mg of crude organellar protein was loaded onto each of the 40 ml gradients (wild type and Δpas8); (b) each gradient was drained into 40 fractions; (c) an equal volume (35 μl) of every fourth fraction (1, 4, 7...40) of each gradient was loaded onto 8 % SDS-PAGE gels (one gel per gradient); (d) these gels were run and transferred to nitrocellulose equivalently; (e) the Western blot filters for each gradient were incubated at the same time and in the same vessels during Western blotting; and (f) for blots with a given antibody, films were developed by ECL for the same amount of time. Film negatives were made from these films with the camera at a fixed setting, and these negatives were printed at the same exposure setting.

Western blots were done using antisera to thiolase (gift of Wolf Kunau, Ruhr-Universitat Bochum, Germany), P. pastoris acyl-CoA oxidase (A. P. Spong and S. Subramaniam, manuscript in preparation), the P. pastoris PAS8p (McCollum et al., 1993) and dihydroxyacetone synthase (DHAS). Antisera to the carboxy-terminal PTS1 peptides, SKL (Gould et al., 1990b) and AKI (gift of R. Rachubinski, University of Alberta, Canada), and antisera to the S. cerevisiae multifunctional enzyme (gift of Wolf Kunau), which recognizes the analogous protein, trifunctional enzyme, of P. pastoris, were also used. In 8 % SDS-PAGE, P. pastoris proteins run at the following molecular masses: thiolase (47 kd), acyl-CoA oxidase (72 kd), trifunctional enzyme (97 kd), PAS8 (68 kd), DHAS (76 kd). The anti-multifunctional enzyme antibody is referred to as anti-trifunctional enzyme antibody. Blots shown in Figs. 9, 10, 11, and 12 were developed using the ECL (Amersham Corp., Arlington Heights, Illinois) system, but all others were done according to Sambrook et al. (1989).

Two-step Sucrose Flotation Gradients

Fractionation of P. pastoris cells into an organellar pellet and supernatant was performed as described above. This pellet was resuspended in 400 μl sucrose gradient buffer, loaded onto a 5-ml sucrose gradient, spun for 2.5 h at 92,000 g, and fractionated into 12 fractions of equal volume. 200 μl of a fraction of density within the range at which purified wild-type peroxisomes are expected to be found (in these gradients, ρ=1.18-1.23 g/cm³) was transferred to the bottom of a Beckman ultra-clear centrifuge tube, overlaid with 2.4 ml 60% sucrose, overlaid again with 2.4 ml 35% sucrose, and the sample was spun at 170,000 g for 18 h and harvested from the bottom (fraction 1) to top (fraction 12 [wild type], fraction 13 [Δpas8]) in fractions of ~0.5 ml (Paravicini et al., 1992). Fractionation gradients were assayed for sucrose density and for catalase and SDH activities across the gradient. These fractions were also immunoprecipitated with the anti-acyl-CoA oxidase, anti-trifunctional enzyme, anti-PAS8, and anti-thiolase antibodies described above. After each flotation gradient had been loaded with a portion of the fraction from each 5-ml purification gradient, each purification gradient was assayed across the gradient for sucrose density and for catalase and SDH activities. The fractions loaded on the flotation gradients had no mito-
chondrial contamination (no SDH activity) and had sucrose densities of 1.21 and 1.20 g/cm³ for the wild-type and Δpas1 fractions, respectively. These fractions were also immunobotted with the anti-acyl-CoA oxidase, anti-trifunctional enzyme, anti-PAS8, and anti-thiolase antibodies to confirm the quality of the purification gradients.

Isolation and Sequencing of the PAS1 Gene

A polymerase chain reaction (PCR) was performed on a P. pastoris genomic DNA library contained in the plasmid pFL20 (Logson and Lacroute, 1983; Gould et al., 1992). Primers "A" and "B", with Universal Wobble Code sequences 5'-GGG GGA TCC NGG NTG YGG NAA RAC NYT NYT NGC-3' and 5'-CCC GAG CTC ARN CKN CCN GGN ARN ARN GC'-3', respectively, were obtained from Operon Technologies, Inc. (Alameda, CA). The PCR product from this reaction was ligated into pUC19, and the resultant plasmids were transformed into DH5α E. coli cells. DNA was isolated from the transformants and sequenced, allowing identification of a clone, pAH11, that contained a fragment of the P. pastoris PAS1. This fragment was subsequently used as a probe to screen by hybridization (performed according to Sambrook et al., 1989) the P. pastoris genomic library and isolate a clone (pAH12) which contained the full-length P. pastoris PAS1 gene.

Sequencing was performed by subcloning fragments into pUC19 (New England Biolabs, Inc., Beverly, MA) and pBluescript/KS II (Strategene, La Jolla, CA). Nested deletions were also generated using Exonuclease III and S1 nuclease as described previously (Sambrook et al., 1989). The Sequenase II system (United States Biochemical, Cleveland, OH) which is based on the dideoxynucleotide chain-termination method was used for nucleotide sequencing. Primers T3 or T7 (for pBluescript-based plasmids) and New England Biolabs primer no. 1212, 1201, 1220, and 1211 (for pUC19-based plasmids) were used together with denatured, double-stranded DNA templates. Both strands were entirely sequenced.

Construction of pas1 Strains

The pas1 disruption (PPY300 [arg4 his4 pas1::ScARG4]) strain was constructed by homologous recombination of the S. cerevisiae ARG4 gene into the P. pastoris PAS1 locus of strain PYPY12. A targeting construct was made in the pUC19 vector by first ligating the 2.4-kb BglIII fragment of PAS1 coding sequence into the BamHI site of pUC19 to create pAH13. Subsequently, the S. cerevisiae ARG4 gene was removed from pSG464 with Sall and blunted-end ligated into the XhoI site of pAH13 to create pAH14. The targeting DNA was released from the vector by digestion with the restriction enzymes Smal and Xbal. The released fragment was isolated from an agarose electrophoresis gel according to Sambrook et al. (1989) and introduced into PYPY12 (arg4 his4) by electroporation (Bio-Rad Laboratories, Hercules, California). (The 2.4-kb fragment and the XhoI site of PAS1 are shown in Fig. 7.)

The pas1 deletion (PPY301 [arg4 his4 Δpas1::ARG4]) was constructed in a similar fashion to that described for PYPY300 except that the targeting construct used is shown in Fig. 7. The plasmid containing the targeting construct (pAH21) was pBluescript/KSII-based, and the targeting DNA was released from this plasmid by the enzymes KpnI and BamHI. Correct targeting of these fragments was confirmed by Southern analysis (Sambrook et al., 1989).

Strain PPY300 was characterized as a peroxisome assembly (pas1) mutant because it did not grow on SM or SOLT, but it did grow on SC, SL, SE, and SG (since yeast require functional mitochondria to utilize glycerol, PPY300 must have functional mitochondria). PPY300 and PPY301 were equally defective for all peroxisomal functions tested.

Complementation of the pas1 Mutants

A 5.2-kb SmaI to BamHI fragment of pAH12 was cloned into the P. pastoris autonomously replicating plasmid pSG464 (Gould et al., 1992). This fragment contained the entire PASI open-reading-frame together with ~900 bp 5' of the initiator ATG and ~500 bp 3' of the stop codon. The resulting plasmid, pAH16, was digested with EcoRV and blunt-end ligated with the 5.2-kb SmaI to BamHI fragment of pAH12 described above. The resulting plasmid, pAH17, was introduced into strains PPY300 and PPY301 by electroporation. Transformants were selected by their ability to grow on plates without arginine. All transformants tested were able to grow on SOLT and SM plates.

In order to complement the PPY300 and PPY301 strains (both histidine auxotrophs), a complementation construct containing the P. pastoris HIS4 gene was constructed. This was accomplished by first blunt-end ligating the P. pastoris PAS2 sequence (Cregg et al., 1985; Gould et al., 1992) into the ClaI site of pYMS, a plasmid consisting of the P. pastoris HIS4 gene cloned into the BamHI site of pBR322. This new construct, pAH18, was digested with EcoRV and blunt-end ligated with the 5.2-kb SmaI to BamHI fragment of pAH12 described above. The resulting plasmid, pAH17, was introduced into strains PPY300 and PPY301 by electroporation. Transformants were selected by their ability to grow on plates without histidine. All transformants tested were able to grow on SOLT and SM plates.

Computer Analysis of Sequences

Sequences were analyzed using the Macvector software (IBI, New Haven, CT) and also FASTA (Pearson and Lipman, 1988).

Results

The Yeast P. pastoris Contains a Homologue of the S. cerevisiae PAS1 Gene

Fig. 1 shows the region of high homology between the S. cerevisiae PAS1 (Erdmann et al., 1991) and S. cerevisiae SEC18 proteins (Eakle et al., 1988). We reasoned that a protein domain shared between these two proteins would also be found in the P. pastoris PAS1 homologue. Accordingly, we designed degenerate PCR primers corresponding to the DNA encoding this domain. Repeatedly, the A and B primers amplified fragments of ~380 and 350 bp from a P. pastoris genomic library. These were the only abundant reaction products seen (data not shown).

As can be inferred from the protein alignment in Fig. 1, a PCR product from the P. pastoris PAS1-homologue DNA

Heyman et al. Role of P. pastoris PAS1 in Peroxisome Biogenesis 1261
Figure 2. Nucleotide sequence and deduced amino acid sequence of the P. pastoris (P.p.) PAS1 gene. PAS1 nucleotide and deduced amino acid sequence. The translation of the PAS1 sequence from the ATG at nucleotide 1 (underlined) to the termination codon is shown in one letter code below the first nucleotide of each codon in the sequence. The two putative ATP-binding domains are double-underlined. The PAS1 open reading frame is 3471 base pairs long, encoding a 1157-amino acid protein with predicted molecular mass of 127 kD.
A comparison of the sequences of the \textit{P. pastoris} PAS1 and the \textit{S. cerevisiae} PAS1 proteins (Fig. 3) shows that the proteins share 39\% sequence identity and 60\% similarity. The \textit{P. pastoris} protein is 114 amino acids longer than the \textit{S. cerevisiae} protein, and regions of strong homology are seen in the line-up at \textit{P. pastoris} residues 458–705 and 771–1052. These regions contain conserved ATP-binding sites at \textit{P. pastoris} residues 523–530 and 840–847, and these are the regions which place PAS1 in the growing family of ATP-binding proteins (Kunau et al., 1993). The family now includes PAS1, CDC48 (Frohlich et al., 1991), and SEC18 (Voorn-Brouwer et al., 1993), from yeasts \textit{P. pastoris}, \textit{Yarrowia lipolytica}, and \textit{S. cerevisiae}, respectively; NSF (the SEC18 homologue [Wilson et al., 1989]), VCP (the CDC48 homologue [Koller and Brownstein, 1987]), and p97 (Peters et al., 1990) from vertebrates; and the virally encoded TBP (Nelbock et al., 1990).

\textit{P. pastoris} PAS1 and \textit{P. pastoris} PAS5 (Fig. 4) share 49\% similarity and 26\% identity. Not surprisingly, the \textit{P. pastoris} \textit{pasl} and \textit{pas5} mutants have similar phenotypes (but they do belong to different complementation groups). These similarities are addressed in the Discussion.

\textbf{Figure 3.} Comparison between \textit{P. pastoris} (\textit{P.p.}) PAS1 and \textit{S. cerevisiae} (\textit{S.c.}) PAS1 proteins. Sequences were aligned using the BEST-FIT program.
As mentioned earlier, the production of peroxisomal proteins required for growth on oleic acid or methanol is greatly increased when *P. pastoris* is grown on either of these carbon sources. It is thought that the increase in size of wild-type peroxisomes during induction is due in part to increased import of these proteins (Hazeu et al., 1975; McCollum et al., 1993). Thus, a defect in peroxisomal protein induction could cause the morphological and growth defects seen in the *P. pastoris* pasl strain.

We tested this possibility by comparing the levels of peroxisomal protein produced in whole-cell lysates of wild-type and *pasl:*ARG4 cells. The *pasl:*ARG4 strain and the wild-type strain produced PAS1 (a peroxisomal matrix protein) at similar levels (Fig. 8). Additionally, catalase and methanol oxidase (both peroxisomal matrix proteins) were induced normally in the *pasl:*ARG4 strain (data not shown). Therefore, PAS1 is not involved in peroxisomal protein induction (data not shown). (These putative "peroxisome ghosts" will be discussed later.)

Thus, the *pasl:*ARG4 disruption confirmed that the PAS1 protein is required for functional peroxisomes in *P. pastoris,* and it defined *P. pastoris* pasl (peroxisome assembly) mutants as being: (a) unable to grow on oleic acid or methanol as sole carbon source; and (b) having no morphologically normal peroxisomes.

Later, to see that the disruption removed all PAS1 activity, we constructed a strain, PPY301 (*arg4* his4 *Δpasl:*ARG4), with a near-complete deletion of the PAS1 gene (Materials and Methods and Fig. 7). The *pasl:*ARG4 and the *Δpasl* strains were equally deficient for all aspects of peroxisomal function tested. (Hereon, PPY301 will be referred to as *Δpasl*.)

We were able to restore functional peroxisomes in both the *pasl:*ARG4 strain (Fig. 6, c and d) and the *Δpasl* strain (data not shown) by transformation with extrachromosomal plasmids that contained the full *P. pastoris* PAS1 open reading frame and ~900 base pairs of DNA 5′ of the ATG initiation codon.

### Peroxisomal Proteins Are Induced Normally in *P. pastoris* Cells

As mentioned earlier, the production of peroxisomal proteins required for growth on oleic acid or methanol is greatly increased when *P. pastoris* is grown on either of these carbon sources. It is thought that the increase in size of wild-type peroxisomes during induction is due in part to increased import of these proteins (Hazeu et al., 1975; McCollum et al., 1993). Thus, a defect in peroxisomal protein induction could cause the morphological and growth defects seen in the *P. pastoris* pasl strain.

We tested this possibility by comparing the levels of peroxisomal protein produced in whole-cell lysates of wild-type and *pasl:*ARG4 cells. The *pasl:*ARG4 strain and the wild-type strain produced thiolase (a peroxisomal matrix protein) at similar levels (Fig. 8). Additionally, catalase and methanol oxidase (both peroxisomal matrix proteins) were induced normally in the *pasl:*ARG4 strain (data not shown). Therefore, PAS1 is not involved in peroxisomal protein induction in *P. pastoris.*

### Δpasl Cells Contain Peroxisomal Ghosts

Erdmann et al. (1991) were unable to find peroxisome-like structures (ghosts) in their *S. cerevisiae* pasl strains by either electron microscopy or by biochemical examination of subcellular fractions. Interestingly, the *P. pastoris* pasl strains contain peroxisomal ghosts.

The electron micrographs in Fig. 6 show *pasl:*ARG4 cells grown on methanol (Fig. 6 a) and oleic acid (Fig. 6 b). In these micrographs we have indicated structures that resemble mutant peroxisomes (McCollum et al., 1993; Spong and Subramani, 1993). These structures are commonly seen in *pasl* cells induced by methanol or oleic acid, but similar structures are only rarely seen in uninduced *pasl* cells or in uninduced *P. pastoris* cells.
uninduced wild-type cells. Interestingly, structures similar to the stacked organelles indicated as peroxisomes in Fig. 6a are regularly seen in P. pastoris Δpas5 cells cultured in methanol media and in wild-type cells cultured in methanol media for short periods (less than 2 h) of time (Heyman and Monosov, unpublished observations).

To further examine the possibility that P. pastoris pasl cells contain mutant peroxisomes, we purified, then compared, peroxisomes from oleic-acid induced wild-type and Δpasl cells. The data presented below were obtained from analysis of two sucrose gradients: The "wild-type" and "Δpasl" gradients were loaded with crude organellar pellets from wild-type and Δpasl cells, respectively. Each 40-ml gradient was loaded with 8 mg total protein. During the characterization of the gradients for Figs. 9 and 10, care was taken in the Western blotting and in the preparation of nega-

Heyman et al. Role of P. pastoris PAS1 in Peroxisome Biogenesis

Figure 5. Growth of P. pastoris on methanol or oleic acid causes a massive proliferation of peroxisomes. (a) Wild-type P. pastoris grown in glucose medium. (b) Wild-type P. pastoris grown in methanol medium. Note the large, clustered peroxisomes (P). (c) Wild-type P. pastoris grown in oleic-acid medium. Note the large, unclustered peroxisomes (P). M, Mitochondria; N, nucleus; P, peroxisome; V, vacuole. Bar, 500 nm.

Figure 6. Methanol- or oleic-acid-induced pasl::ARG4 cells contain structures that look like very small peroxisomes. Transformation with extrachromosomal copies of the PAS1 gene restores normal peroxisomes to the pasl::ARG4 strain. (a) pasl::ARG4 cells cultured in methanol medium contain clustered, single-membrane-bound structures that resemble small peroxisomes (P). (b) pasl::ARG4 cells cultured in oleic-acid medium contain several small, single membrane-bound structures that are not seen in uninduced wild-type cells. pasl:: ARG4 cells harboring the PAS1 gene on extrachromosomal plasmids contain morphologically wild-type peroxisomes (P) when cultured in (c) methanol, or (d) oleic-acid. Bar, 500 nm.
Figure 7. Deletion of the PAS1 gene. The 5' 600-bp EcoRI (R) fragment (blunted) and the 3' 1200-bp BgIII (G) to BamHI (B) fragment were cut from pIAH15 (a) and cloned consecutively into the XhoI (blunted and treated with phosphatase) and BamHI (treated with phosphatase) sites, respectively, of the pBluescript-IIIKS multiple cloning site (mcs), forming the construct in b. The P. pastoris ARG4 gene was then excised from pAS100 (Spong and Subramani, 1993) with KpnI and EcoRV (a 2075-bp fragment), blunted, and ligated into the EcoRV (treated with phosphatase) site between the PAS1 fragments (c). Thus, 2.4 kb of PAS1 coding sequence was replaced with the P. pastoris ARG4 gene. Digestion at the KpnI (K) and Scal (S) sites was used to release the PAS1-targeted ARG4 gene, and this DNA was introduced into the genome by homologous recombination into PPY12, forming the Δpas1 locus depicted in d. Enzyme sites in brackets were destroyed during cloning. The PAS1 coding sequence is shaded and bounded by heavy vertical lines.

Figure 8. Growth on oleic acid or methanol causes normal induction of peroxisomal proteins in pas1::ARG4 cells. Wild-type and pas1::ARG4 cells were precultivated in yeast nitrogen base with 2% glucose (SG) and then shifted to either yeast nitrogen base with methanol (SM) or yeast nitrogen base with oleic acid and Tween (SOLT) for 1.5, 3.5, or 7.5 h. (a) The methanol-induced cells were immuno-blotted for the presence of DHAS and PAS8. (b) The oleic-acid-induced cells were immuno-blotted for the presence of PAS8 and thiolase.

Fig. 9 summarizes experiments performed on sucrose fractions from a peroxisome purification gradient for wild-type cells. Catalase activity was seen primarily in fractions 13–25, with a peak in fraction 16, and SDH activity (mitochondrial marker) was seen in fractions 28–37, with peak activity in fraction 31 (Fig. 9a). Western blots with antibodies against trifunctional enzyme (Fig. 9b), acyl-CoA oxidase (Fig. 9c), and thiolase (Fig. 9d) show that these peroxisomal proteins also colocalized with catalase within the gradient. Blotting with anti-PAS8 antibody (Fig. 9e) showed the PAS8 protein to have a bimodal distribution within the gradient: one peak coincided with the peroxisomal markers, and the other peak was likely to be the result of PAS8 protein running as a free protein or as a protein that sticks nonspecifically to mitochondria. (An experiment in which human serum albumin was added to an organelle pellet before sucrose gradient centrifugation showed that free protein runs at the top of these sucrose gradients; data not shown.)

Since four peroxisomal matrix proteins and one peroxisomal membrane-associated protein colocalized in fractions 13–25, and because there was virtually no SDH (Fig. 9a) activity in these fractions, it is clear that intact peroxisomes, free of mitochondrial contamination, were purified in frac-
Figure 9. Purification of peroxisomes from wild-type cells on a sucrose gradient. Wild-type cells were induced in oleic acid for 18 h and the organelle pellet fraction (8 mg), consisting primarily of peroxisomes and mitochondria, was fractionated on a sucrose gradient as described by Nuttley et al. (1990). The gradient was drained into 40 1-ml fractions. (a) Gradient fractions assayed for catalase and SDH (succinate dehydrogenase) activity across the gradient. Catalase activity is expressed in Beaufay U/ml. SDH activity is expressed as the change in absorbance at 410 nm/min/ml of fraction. Density of fractions in g/cm³. (b-e), results of Western blotting equal volumes (35 µl) of every fourth gradient fraction (including the first and last fractions) with: (b) antisera to trifunctional enzyme (abbreviated "anti-TFE"); (c) antisera to acyl-CoA oxidase (abbreviated "anti-AOX"); (d) antisera to thiolase; and (e) affinity-purified antisera to P. pastoris PAS8. Antibody dilutions were 1/2,000, 1/1,000, 1/500, 1/1,000, respectively.

Figure 10 summarizes experiments performed on sucrose gradient fractions from a peroxisome purification gradient from Δpas1 cells. Similar to the wild-type gradient, SDH activity was found in fractions 28–37, with peak activity in fraction 31 (Fig. 10 a). Catalase activity (Fig. 10 a), however, was not found at all in fractions 13–25, and the only detectable catalase was a small amount found in fractions 37–40 (corresponding to free protein). An immunoblot with anti-thiolase antibody (Fig. 10 d) demonstrated that no thiolase was loaded on the gradient (even a very long ECL exposure did not show any thiolase on this blot; data not shown). These results suggest that the Δpas1 cells did not import thiolase and catalase into membrane-bound structures.

Blotting Δpas1 gradient fractions with antibodies that recognize the trifunctional protein (Fig. 10 b) and acyl-CoA oxidase
oxidase protein (Fig. 10 c) revealed that both proteins concentrate in two distinct parts of the gradient: fractions 34–40, which are likely to contain free protein, and fractions 13–19, which correspond (both in number and in sucrose density) to the peak peroxisome-containing fractions from the wild-type gradient (Fig. 9). A blot with anti-PAS8 antibody (Fig. 10 e) shows that the PAS8 protein was distributed through the gradient in a bimodal manner, as was seen for the wild-type gradient. The comigration of acyl-CoA oxidase, PAS8 protein, and the trifunctional enzyme to fraction 16 is striking, especially in light of the fact that both the wild-type gradient fraction 16 (peak peroxisomal fraction) and Δpasl fraction 16 have a density of 1.19 g/cm³.

The colocalization of these peroxisomal markers to fractions of 1.19 g/cm³ sucrose density in the gradient from Δpasl cells was most likely due to one of three reasons: (a) the three proteins were part of a large protein complex that had sedimented to sucrose of 1.19 g/cm³ during the 8-h, 92,000-g spin; (b) the three proteins were loaded onto the sucrose gradient as a result of nonspecific clinging to the crude pellet, then during the spin migrated as free proteins to sucrose of density 1.19 g/cm³; or (c) the three proteins are associated with peroxisome ghosts.

To distinguish between the first and third possibilities, we performed a sucrose-flotation experiment (Walworth et al., 1989; Paravincini et al., 1992). Crude organellar pellets were prepared from oleic acid-induced wild-type and Δpasl cells, then loaded on a 5-ml sucrose gradients and spun for 2.5 h at 92,000 g. These gradients were fractionated and 200-μl gradient material of the appropriate peroxisome- or peroxisome ghost-containing fraction was placed in a 5-ml high-speed centrifuge tube, overlaid with cushions (2.4 ml) of 60 and 35% sucrose and centrifuged at 170,000 g for 18 h in a swinging bucket rotor. Fractions were then assayed for the presence of peroxisomal markers. It was expected that protein aggregates that did not completely sediment during the first (8 h, 92,000 g) spin would completely sediment during the second (18 h, 170,000 g [Horazdovsky and Emr, 1993]) spin, and that membrane-enclosed proteins would rise to the 60/35% sucrose interface (Stack et al., 1993). Since no mitochondria were loaded onto the flotation gradient (the material loaded onto this gradient contained no SDH activity; data not shown), there was no chance that proteins could rise to the 60/35% sucrose interface due to interaction with mitochondrial membranes.

In both wild-type and Δpasl gradients, it is clear that the peroxisomal markers (trifunctional enzyme, acyl-CoA oxidase, and PAS8 protein) floated out of the densest sucrose and concentrated at the interface of the 60 and 35% sucrose (Figs. 11 and 12).

The fact that the peroxisomal markers behave similarly in the wild-type and the Δpasl flotation gradients suggests that the colocalization of peroxisome markers in the Δpasl purification gradient was not the result of the proteins being in a complex that had sedimented to sucrose of 1.19 g/cm³ during the 8-h, 92,000-g spin.

To test the possibility that acyl-CoA oxidase, trifunctional enzyme, and PAS8 migrated as free proteins to sucrose of density 1.19 g/cm³ in the Δpasl purification gradient, we ran sucrose purification gradients with organellar pellets prepared from P. pastoris wild-type, Δpasl, and Δpas8 cells. The Δpas8 strain was used because it is known that it will not import acyl-CoA oxidase or trifunctional enzyme into peroxisomes: P. pastoris Δpas8, and the equivalent S. cerevisiae strain, paslo, are characterized by peroxisomes that fail to import PTS1-containing proteins and the PAS8 protein of P. pastoris is the PTS1 receptor (McCollum et al., 1993; van der Leij et al., 1993).

In the wild-type and the Δpasl gradients, trifunctional enzyme and acyl-CoA oxidase were distributed essentially as in the gradients described in Figs. 9 and 10 (peroxisomal markers were found in peak levels in both gradients at density 1.21 g/cm³; data not shown) whereas in the Δpas8 gradient, no acyl-CoA oxidase was detected at all, and the very small amount of trifunctional enzyme detected was found at a density of 1.16 g/cm³ at the top of the gradient. Thus, in Δpas8 cells, acyl-CoA oxidase was not imported into peroxisomes, nor did it cling to the pre-gradient pellet in an amount

**Figure 11.** Localization of peroxisomal markers in two-step sucrose gradients. Wild-type cells were induced on oleic acid for 18 h and an organellar pellet fraction, consisting primarily of peroxisomes and mitochondria, was obtained. This pellet was loaded on a 5-ml sucrose gradient, spun for 2.5 h at 92,000 g, and fractionated into 12 fractions of ~480 μl each. 200 μl from a fraction of density 1.21 g/cm³ was transferred to the bottom of a Beckman ultra-clear centrifuge tube, overlaid with 2.4 ml 60% sucrose, then overlaid again with 2.4 ml 35% sucrose. This sample was centrifuged at 170,000 g for 18 h and dripped from bottom (fraction 1) to top (fraction 12) in ~0.5 ml fractions. (a) Gradient fractions assayed for SDH activity across the gradient. Density of fractions in g/cm³. (b–d) Equval equal volumes (35 μl) of fractions Western-blotted with the indicated antisera. Antibody dilutions are as described in the legend to Fig. 9.
great enough to allow its detection in theΔpas8 sucrose gradient. The small amount of trifunctional enzyme that did get loaded on theΔpas8 gradient did not migrate to the density of wild-type peroxisomes. These results rule out the possibility that the distribution of trifunctional enzyme and acyl-CoA oxidase seen in theΔpas1 sucrose gradient described in Fig. 10 was due to the two proteins clinging nonspecifically to the crude pellet and then migrating as free proteins to sucrose of density 1.19 g/cm³. This fact, coupled with the flotation gradient results, suggests that trifunctional enzyme and acyl-CoA are imported to membrane-bound structures inΔpas1 cells. Furthermore, these membrane-bound structures are peroxisomes because they have the correct density and they contain several peroxisomal markers, but no SDH activity.

Δpas1 Peroxisomal Ghosts Contain Some, but Not All, of the Proteins Seen in Wild-type Peroxisomes

When Figs. 9 and 10 are compared, it can be seen thatΔpas1 peroxisomal ghosts were completely deficient for the import of thiolase and catalase. When care is taken (see Materials and Methods) to ensure that the band intensity of a particular marker protein in gradient fractions can be used to compare the relative levels of that protein in theΔpas1 and wild-type gradients, it is clear that several proteins (trifunctional enzyme, PAS8 protein, and acyl-CoA oxidase) were indeed imported to theΔpas1 cells, but at very low levels when compared to the levels seen in wild-type peroxisomes. This is well illustrated by the difference in band intensity seen for trifunctional enzyme in Western blots of the wild-type (Fig. 9 b) andΔpas1 (Fig. 10 b) sucrose gradients (ECL development time required to detect trifunctional enzyme on theΔpas1 blot is sufficient to overexpose the band corresponding to this protein in the wild-type blot).

Fractions from wild-type andΔpas1 gradients were also blotted with anti-SKL antibody. Since COOH-terminal -SKL tripeptides are used as peroxisomai targeting signals in yeast (Distel et al., 1992), it was expected that antibodies directed against these sequences would recognize proteins in purified wild-type peroxisomes. This was the case: four proteins much enriched in the peroxisomal fractions were recognized by anti-SKL antibody (molecular masses: 97, 72, 38, and 31 kD). Interestingly, when fractions from aΔpas1 sucrose gradient were blotted with anti-SKL antibody, only the 97-kD protein (now known to be trifunctional enzyme) and the 72-kD protein (now known to be acyl-CoA oxidase) were detected. The two other proteins (38 and 31 kD) recognized by the anti-SKL antibody were not detected in theΔpas1 gradient (data not shown). However, since these two proteins are seen at lower intensities than trifunctional enzyme or acyl CoA oxidase in blots of wild-type peroxisomes, it is possible that small amounts of these proteins might have gone undetected in the peroxisomes fromΔpas1 cells.

Thus peroxisome ghosts fromΔpas1 cells are unable to import any of the proteins tested with the same efficiency as wild-type peroxisomes. Some peroxisomal proteins (thiolase, catalase and the 38- and 31-kD proteins) are undetectable in the organelles fromΔpas1 cells, but others (trifunctional enzyme, acyl CoA oxidase, and PAS8) are present at reduced levels, relative to that in wild-type cells.

It is interesting that the proteins found in association withΔpas1 ghosts include both PTS1-targeted proteins (trifunctional enzyme, acyl-CoA oxidase) and at least one protein that is not targeted via this pathway (PAS8 has no PTS1 sequence [McCullum et al., 1993]).Δpas1 cells appear to be deficient in the import of a PTS2-containing protein (thiolase), as well as some proteins that are either known to be imported via the PTS1 pathway (e.g., catalase, McCollum et al., 1993) or others that are recognized by the anti-SKL antibody and may therefore be imported via this pathway.

The PAS1 Protein Is Induced by Growth on Oleic Acid or Methanol

Crude P. pastoris lysates from glucose-grown cells were blotted with an antibody made against a GST-PAS1 fusion protein. A protein of the predicted size, 127 kD, was recognized (Fig. 13). This protein was seen in increased amounts in lysates from cells grown on oleic acid or methanol. This protein was not recognized in lysates from pasl::ARG4 cells grown on glucose, oleic acid, or methanol, proving that the
The evidence that the \(P.\) \textit{pastoris} \(PAS1\) gene is required for peroxisomal biogenesis and is the homologue of the \(S.\) \textit{cerevisiae} \(PAS1\) is as follows. 

(a) Disruption or deletion (by homologous recombination) of the \(P.\) \textit{pastoris} \(PAS1\) results in cells that are unable to grow on oleic acid or methanol, carbon sources known to cause induction of wild-type \(P.\) \textit{pastoris} peroxisomes and peroxisomal enzymes. In \(S.\) \textit{cerevisiae}, oleic acid is the only carbon source known to induct peroxisomes and peroxisomal proteins (Kunau et al., 1988), and \(S.\) \textit{cerevisiae} \(pasl\) cells are unable to grow on oleic acid as sole carbon source (Erdmann et al., 1989). 

(b) Electron micrographs of \(P.\) \textit{pastoris} \(pasl\) cells induced on oleic acid or methanol show fewer and much smaller peroxisome-like structures than do similarly grown wild-type \(P.\) \textit{pastoris} cells (Fig. 5, \(b\) and \(c\)). This is similar to results seen for \(S.\) \textit{cerevisiae}, in which peroxisomal structures are never seen in oleic acid-induced \(pasl\) cells but are easily observed in induced wild-type cells. 

(c) Comparison of the predicted amino acid sequences reveals that the \(P.\) \textit{pastoris} \(PAS1\) shares 60\% similarity and 39\% identity with the \(S.\) \textit{cerevisiae} \(PAS1\) (Fig. 3). The sequences contain 1157 and 1043 residues, respectively, with homology throughout the length of the proteins and extremely high homology in two regions containing consensus ATP-binding domains.

Additionally, a search of GENBANK with the predicted \(P.\) \textit{pastoris} \(PAS1\) sequence reveals \(S.\) \textit{cerevisiae} \(PAS1\) as its best match in GENBANK. For comparison, \(P.\) \textit{pastoris} \(PAS8\) (McCullum et al., 1993) and its homologue, \(S.\) \textit{cerevisiae} \(PAS10p\), share 57\% similarity and 37\% identity (van der Leij et al., 1993). 

(d) Introduction of the cloned \(P.\) \textit{pastoris} \(PAS1\) gene complements the peroxisomal defects seen in \(P.\) \textit{pastoris} strains bearing a disruption or deletion of the chromosomal \(PAS1\) gene. 

(e) In Western blots, an antibody made against a GST-PAS1 fusion protein reacts with a protein of 127 kD. This protein is the correct size, it is induced on oleate and methanol (Fig. 13), it is absent in strains bearing a deleted or disrupted chromosomal \(PAS1\) gene, and it is over-expressed in strains bearing the \(PAS1\) gene on a multicopy episomal plasmid (relative to the amount of \(PAS1\) seen in similarly grown wild-type cells; data not shown). 

(f) The \(PAS1\) genes of \(P.\) \textit{pastoris} and \(S.\) \textit{cerevisiae} are more closely related to each other (Fig. 3) than the \(PAS1\) and \(PAS5\) genes of \(P.\) \textit{pastoris} (Fig. 4) which complement mutations that belong to different complementation groups, yet encode related proteins.

Discussion

The \(P.\) \textit{pastoris} \(PAS1\) is a Homologue of the \(S.\) \textit{cerevisiae} \(PAS1\) and Is Required for Functional Peroxisomes

Electron micrographs of oleic acid- or methanol-induced \(Delta pasl\) cells contain structures that look like extremely small peroxisomes (Fig. 6, \(a\) and \(b\)) which are quite distinct morphologically compared to the peroxisomes seen under similar circumstances in wild-type cells (Fig. 5, \(b\) and \(c\)).

To confirm that \(Delta pasl\) cells contain peroxisomal "ghosts," we purified these structures on sucrose gradients. Several peroxisome markers (acyl-CoA oxidase, trifunctional protein, and \(PAS8\)) comigrated to fractions of the same sucrose density (1.19 g/cm\(^3\)) to which wild-type peroxisomes and peroxisomal markers migrate (Figs. 9 and 10). The colocalization, in \(Delta pasl\) sucrrose gradients, of bona fide peroxisomal markers at the same sucrose density as that of wild-type peroxisomes was not fortuitous. This was shown by the fact that both acyl-CoA oxidase and trifunctional enzyme, which in wild-type \(P.\) \textit{pastoris} are imported into peroxisomes via the PTS1 pathway and migrate in sucrose organellae purification gradients to a density of 1.19 g/cm\(^3\), failed to localize
to this density in sucrose gradients loaded with an organellar pellet isolated from PTS1-import deficient Δpas8 cells (data not shown). The colocalization of multiple peroxisomal markers at a sucrose density of 1,19 g/cm² in Δpasl cells cannot be due to simple aggregation of these proteins either, as demonstrated by the fact that the markers floated to the interface between 60 and 35% sucrose during the Δpasl sucrose flotation gradient experiment (Fig. 12) in a fashion virtually identical to that seen for markers enclosed in wild-type peroxisomes (Fig. 11). These observations provide strong evidence for the association and import of acyl CoA oxidase and trifunctional enzyme into peroxisome ghosts of Δpasl cells. The presence of PAS8, the putative PTS1 receptor, in peroxisome ghosts of Δpasl cells is consistent with their ability to import small quantities of certain PTS1-containing proteins.

The existence of detectable peroxisome ghosts in the Δpasl mutant of Ρ. pastoris clearly distinguishes it from the analogous mutant of S. cerevisiae (Erdmann et al., 1991). It is possible that these ghosts are more visible by electron microscopic analysis and more stable during biochemical manipulations in Ρ. pastoris, relative to S. cerevisiae. The existence of these ghosts however, provides a satisfying explanation for the fact that peroxisomes reappear in the pasl mutants complemented with the PASl gene from the corresponding organism (Fig. 6, c and d). If peroxisomes arise only from preexisting peroxisomes, and if the pasl mutant of S. cerevisiae really has no peroxisome ghosts, then it would be difficult to explain how peroxisomes are restored by the introduction of the PASI gene into the pasl mutant of S. cerevisiae.

Δpasl Peroxisomal Ghosts Import Some, but Not All, of the Proteins Seen in Wild-type Peroxisomes

The phenotype of the S. cerevisiae pasl mutant, i.e., cells with no detectable peroxisomes, has been explained as the result of a defect in protein import into peroxisomes (Erdmann et al., 1991). A more detailed analysis of the pasl mutant of Ρ. pastoris shows that the situation is more complex. A comparison of data obtained from Western blots of wild-type and Δpasl peroxisomal purification gradient fractions confirmed that Δpasl cells contain ghosts, and that these ghosts did not contain wild-levels of peroxisomal proteins (Figs. 9 and 10). Interestingly, however, a peroxisomal protein's import-fate in Δpasl does not seem to be dependent on the protein's targeting signal alone: proteins absent from Δpasl peroxisomes include the PTS1-targeted catalase and the PTS2-targeted thiolase, while proteins that are imported into Δpasl peroxisomes include two PTS1-targeted proteins (trifunctional enzyme, which is recognized by anti-SKL antibody, and acyl-CoA oxidase, which is recognized by anti-AKI antibody and anti-SKL antibody) and PAS8, which does not contain a PTS1 or PTS2 (McCollum et al., 1993).

These results argue against PASI having a direct role in the import of proteins bearing a specific type of peroxisomal targeting signal: if this were the case, it would be expected that Δpasl peroxisomes would contain wild-type amounts of all proteins except those targeted by a PASI-requiring pathway. We have not yet determined the PASI protein's cellular location, but cellular fractionation suggests that PASI is not a free cytosolic protein. Additionally, Western blots of sucrose-gradient purified peroxisomes isolated from oleic acid-induced, wild-type Ρ. pastoris revealed no PASI protein associated with peroxisomes.

Role of the PASI Protein in Peroxisome Biogenesis

Ρ. pastoris pasl cells contain extremely small peroxisomes that will not support growth on oleic acid or methanol. The fact that Δpasl cells contain peroxisomal proteins suggests that Ρ. pastoris PASI is not required for distribution of peroxisomes to daughter cells. Additionally, pasl::ARG4 cells (which are phenotypically equivalent to Δpasl cells) produce peroxisomal proteins at wild-type levels (Fig. 8), suggesting that PASI is not involved in the induction of peroxisomal proteins. Further, it is unlikely that a defect in peroxisomal substrate import would result in extremely small peroxisomes. Thus, the Δpasl phenotype is likely to be due to a defect in peroxisomal matrix protein import or in the addition of membrane to growing peroxisomes.

The following characteristics of the Δpasl ghosts lead us to believe that PASI functions in the addition of membrane to peroxisomes: (a) electron micrographs reveal that induced Δpasl cells often contain structures that look like small peroxisomes. Similar structures are rarely seen in uninduced or fully induced wild-type cells. Interestingly, structures similar to the small, stacked organelles seen in the methanol-cultured Δpasl cells (Fig. 6 a) are regularly seen in methanol-cultivated Δpas5 cells and in wild-type cells cultivated for short periods (less than 2 h) in methanol. This suggests that the peroxisome ghosts seen in the Δpasl cells may be normal intermediates in the peroxisome biogenesis pathway and the situation is reminiscent of the immature acyl CoA oxidase-containing peroxisomes of rat liver cells, which import other proteins to become mature peroxisomes (Heinemann and Just, 1992; Luers et al., 1993). (b) Peroxisomal ghosts isolated from Δpasl cells contain several, but not all, of the proteins seen associated with wild-type peroxisomes. Since peroxisomal proteins of at least two types (matrix proteins targeted via the PTS1 pathway and at least one membrane protein [PAS8] targeted via a different signal) are associated with purified ghosts, it does not seem likely that pasl cells are simply defective for matrix protein import. It should be noted that Δpasl cells have fewer and smaller peroxisomes than wild-type cells under analogous peroxisome-induction conditions. The reduced efficiency of import of markers such as acyl-CoA oxidase, trifunctional enzyme or PAS8p, into peroxisomes of Δpasl cells could be due, at least in part, to the possibility that these organelles can only accommodate limited amounts of matrix proteins. If they become full, and if the organelle cannot grow in size due to some other limitation, then there would appear to be a protein import defect.

These results are consistent with PASI playing a role in membrane addition: if peroxisomes could import protein but not acquire membrane material such as lipid or protein, cells growing on oleic acid or methanol would soon fill their peroxisomes to capacity with protein. Also, if it is posited that different peroxisomal proteins are imported to or retained in peroxisomes at different efficiencies, then peroxisomal proteins would be expected to collect in peroxisomal ghosts at differing levels.

A membrane-addition role for the PASI protein is consistent with the results found for S. cerevisiae pasl. This mutant
had no detectable peroxisomes, but Erdmann et al. (1991) mentioned that diminished peroxisomes might have been present and simply not detected. Additionally, they stated that the S. cerevisiae pas1 defect could be complemented by the addition of the PAS1 gene and that, if S. cerevisiae pas1 cells contained no peroxisomes, this result would be inconsistent with the dogma that peroxisomes must come from preexisting peroxisomes.

It was mentioned earlier that P. pastoris PAS1 and PAS5 (Spong and Subramani, 1993) share considerable homology. Thus, it should be possible to explain the pas5 phenotype as the result of a defect in membrane addition. This is the case because the pas5 phenotype is similar to the pas1 phenotype: cells from each strain contain small (with respect to wild type) peroxisomes and these peroxisomes can import some peroxisomal proteins (Spong and Subramani, 1993) but not others. Thus, the results reported for the pas5 mutant are not inconsistent with a peroxisomal membrane addition role for PAS5.

Finally, the fact that SEC18 (Eakle et al., 1988) and its mammalian homologue NSF (Wilson et al., 1989) are thought to play a role in the intracellular trafficking of membrane-bound vesicles suggests that PAS1 may also be involved in intracellular membrane movement.

We thank Jon Singer for his help and support of E. Monosov. We thank Scott Emr for advice and Wolf Kunau for sharing the S. cerevisiae PAS1 gene and sequence with us. We thank Rick Rachubinski for the anti-AK1 antibody and Wolf Kunau for the anti-multi-enzymatic enzyme and anti-thiolase antibodies. We thank Steve Gould for his help in designing the PCR oligos, and Dan McCollum and Allan Spong for helpful insights.

This work was supported by a Guggenheim fellowship and by a National Institutes of Health grant (GM 15971) to J. Singer. E. Monosov was supported by National Institutes of Health grant (GM 15971) to J. Singer.

Received for publication 27 January 1994 and in revised form 12 September 1994.
Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell. Biol.* 9: 445–478.
Van den Bosch, H., R. B. H. Schutgens, R. J. A. Wanders, and J. M. Tager. 1992. Biochemistry of peroxisomes. *Annu. Rev. Biochem.* 61: 157–197.
van der Leij, I., M. Franse, Y. Elgersma, B. Distel, and H. F. Tabak. 1993. PAS10 is a tetrapeptide-repeat protein that is essential for the import of most matrix proteins into peroxisomes of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 90: 11782–11786.
Veenhuis, M., J. P. van Dijken, S. A. Pilon, and W. Harder. 1978. Development of crystalline peroxisomes in methanol-grown cells of the yeast *Han senula polymorpha* and its relation to environmental conditions. *Arch. Microbiol.* 117: 153–163.
Voorn-Brouwer, T., J. van der Leij, W. Hemrika, B. Distel, and H. F. Tabak. 1993. Sequence of the PAS8 gene, the product of which is essential for biogenesis of peroxisomes in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta.* 1216: 325–328.
Watworth, N. C., B. Goud, H. Ruohoala, and P. J. Novick. 1989. Fractionation of yeast organelles. *Methods Cell Biol.* 31: 335–356.
Wilson, D. W., C. A. Wilcox, G. C. Flynn, E. Chen, W.-J. Kuang, W. J. Henzel, M. R. Block, A. Ullrich, and J. E. Rothman. 1989. A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature (Lond.)*. 339: 355–359.
Zhou, C., Y. Yang, and A. Y. Hong. 1990. Mini-prep in ten minutes. *Biotechniques.* 2: 172–173.