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Formation of the Peroxisome Lumen Is Abolished by Loss of *Pichia pastoris* Pas7p, a Zinc-Binding Integral Membrane Protein of the Peroxisome

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We have cloned and sequenced *PAS7*, a gene required for peroxisome assembly in the yeast *Pichia pastoris*. The product of this gene, Pas7p, is a member of the C_3HC_4 superfamily of zinc-binding proteins. Point mutations that alter conserved residues of the C_3HC_4 motif abolish *PAS7* activity and reduce zinc binding, suggesting that Pas7p binds zinc in vivo and that zinc binding is essential for *PAS7* function. As with most *pas* mutants, *pas7* cells exhibit a pronounced deficiency in import of peroxisomal matrix proteins that contain either the type 1 peroxisomal targeting signal (PTS1) or the type 2 PTS (PTS2). However, while other yeast and mammalian *pas* mutants accumulate ovoid, vesicular peroxisomal intermediates, loss of Pas7p leads to accumulation of membrane sheets and vesicles which lack a recognizable lumen. Thus, Pas7p appears to be essential for protein translocation into peroxisomes as well as formation of the lumen of the organelle. Consistent with these data, we find that Pas7p is an integral peroxisomal membrane protein which is entirely resistant to exogenous protease and thus appears to reside completely within the peroxisome. Our observations suggest that the function of Pas7p defines a previously unrecognized step in peroxisome assembly: formation of the peroxisome lumen. Furthermore, because the peroxisomal intermediates in the *pas7*Δ mutant proliferate in response to peroxisome-inducing environmental conditions, we conclude that Pas7p is not required for peroxisome proliferation.

Peroxisomes are single-membrane bound organelles common to almost all eukaryotes. Certain metabolic functions are common to most peroxisomes, chief among these being the β-oxidation of fatty acids and the consumption of H_2O_2 by catalase. More specialized peroxisomal functions include the synthesis of ether lipids, bile acids, and cholesterol in mammalian tissues, the glyoxylate cycle in plants and fungi, yeast methanol oxidation, and glycolysis in the trypanosomatids (76, 80). Unlike the mitochondrion, the nucleus, and organelles of the secretory pathway (the endoplasmic reticulum, Golgi apparatus, secretory vesicles, etc.), the peroxisome is not essential for cell survival, since mutants defective in peroxisome assembly are viable (71). Nevertheless, the peroxisome is essential for normal human development, since defects in peroxisome assembly are the cause of the peroxisome biogenesis disorders (PBDs) (45): Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum's disease, and rhizomelic chondrodysplasia punctata (68). These diseases are characterized by severe developmental abnormalities, particularly within the nervous system, and usually result in death in early infancy.

Peroxisomes are thought to arise by budding or fission from preexisting peroxisomes (44), although evidence for de novo synthesis has been presented (84). Both peroxisomal membrane proteins and peroxisomal matrix proteins are synthesized on free polyribosomes and imported posttranslationally (44). All peroxisomal proteins contain *cis*-acting peroxisomal targeting signals (PTSs) which are required for their transport to and into the peroxisome (71). Of these, PTS1 (1, 4, 11, 27–31, 34, 41, 54, 73) and PTS2 (22, 25, 57, 72) target proteins only to the peroxisome lumen and are the most completely characterized. Because the sequence of these signals appears to vary considerably from one species to another, it may be more appropriate to define PTS1-containing proteins as those which are imported by the PTS1 receptor and PTS2-containing proteins as those which are imported by the putative PTS2 receptor. In addition, a targeting signal for a peroxisomal membrane protein has been reported (51). Regarding the import process itself, it has been shown that ATP (38, 85) and cytosolic factors (85) are required. In addition, Glover et al. (24) and McNew and Goodman (53) have demonstrated that proteins can be imported into peroxisomes in an oligomeric state.

To further our understanding of peroxisome biogenesis, we have undertaken a genetic analysis of peroxisome assembly in the yeast *Pichia pastoris*. In this yeast, certain enzymes required for growth on fatty acids (enzymes of the β-oxidation spiral) and methanol (alcohol oxidase and dihydroxyacetone synthase) are located within peroxisomes. Furthermore, these enzymes must be compartmentalized within peroxisomes for the cell to utilize these carbon and energy sources. Mutants defective in peroxisome assembly (*pas* mutants) are specifically unable to grow on fatty acids or methanol, lack morphologically normal peroxisomes, and are unable to import one or more classes of peroxisomal matrix proteins from the cytoplasm. We and others have used *pas* mutants of *P. pastoris* (32, 47), *Saccharomyces cerevisiae* (13, 14), *Yarrowia lipolytica* (56), and *Hansenula polymorpha* (9) to identify several genes that are required for peroxisome assembly (8, 15, 35, 37, 50, 52, 56, 69, 74, 81–83, 86). In addition to providing us with a more detailed

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
PPY26	*arg4-1 pas7-1*	32
SGY21	*arg4-1 pas1*Δ::*ARG4 his4*Δ::*ARG4*	26
SGY22	*arg4-1 pas2-1 his4*Δ::*ARG4*	26
SGY23	*arg4-1 pas5-1 his4*Δ::*ARG4*	26
SGY24	*arg4-1 pas6*Δ::*ARG4 his4*Δ::*ARG4*	26
SGY25	*arg4-1 pas8-1 his4*Δ::*ARG4*	26
SGY26	*arg4-1 pas9-1 his4*Δ::*ARG4*	26
SGY27	*arg4-1 pas11-1 his4*Δ::*ARG4*	26
SGY53	*arg4-1 leu2*Δ::*ARG4*	87
SGY55	*arg4-1 his4*Δ::*ARG4*	26
SGY101	*arg4-1 pas4*Δ::*ARG4 his4*Δ::*ARG4*	8
SGY130	*arg4-1 leu2*::*ARG4 pas7*Δ::*LEU2*	This study
SGY131	*arg4-1 pas7*Δ::*LEU2*	This study
SGY443	*arg4-1 pas10*Δ::*ARG4 his4*Δ::*ARG4*	40
SGY741	*arg4-1 pas7*Δ::*LEU2 his4*Δ::*ARG4 (pSG741)*	This study
SGY746	*arg4-1 pas7*Δ::*LEU2 his4*Δ::*ARG4* (pSG746)	This study
SGY747	*arg4-1 pas7*Δ::*LEU2 his4*Δ::*ARG4* (pSG747)	This study
SGY748	*arg4-1 pas7*Δ::*LEU2 his4*Δ::*ARG4* (pSG648)	This study
SGY749	*arg4-1 pas7*Δ::*LEU2 his4*Δ::*ARG4* (pSG749)	This study

understanding of peroxisome assembly, these analyses are helping us to elucidate the molecular basis of the peroxisome biogenesis disorders in humans: we have recently found that *PXR1*, the human homolog of the *P. pastoris PAS8* and *S. cerevisiae PAS10* genes, is essential for peroxisome assembly in humans (12). Furthermore, this gene is mutated in all patients with complementation group 2 of the PBDs (12). We report here on the characterization of the *P. pastoris PAS7* gene and its product, Pas7p. We find that the loss of Pas7p does not interfere with the formation of peroxisome membranes or with the process of peroxisome proliferation. However, Pas7p does appear to be required for import of matrix proteins containing either type of PTS as well as for the elaboration of the peroxisome lumen.

MATERIALS AND METHODS

Strains. *Escherichia coli* DH10B (33) was used for almost all procedures involving bacteria, and strain BL21(DE3) (70) was used for expression of the pRSET-Pas7p fusion protein. The yeast strains used in this study are listed in Table 1. Plasmids were introduced into *P. pastoris* strains by electroporation, as described previously (8). All media and growth conditions for *P. pastoris* are described by Gould et al. (32) and Crane et al. (8).

Isolation and nucleotide sequencing of the *PAS7* gene. A *P. pastoris ARG4*-based genomic DNA library (32) was introduced into strain PPY26. ARG^+ transformants were obtained and transferred to minimal methanol medium to select for complementation of the *pas7-1* mutant. Total DNA was isolated from PAS^+ clones (36) and electroporated into *E. coli*. Plasmid DNAs able to complement the *pas7-1* mutant upon reintroduction into PPY26 were subcloned into *ARG4*-based *E. coli-P. pastoris* shuttle vectors (26) and tested for complementing activity. The smallest complementing clone was sequenced but turned out to lack a complete open reading frame (ORF) for the complementing gene (complementation was due to gene conversion repair of the *pas7-1* allele rather than action of a truncated Pas7p molecule). A 1.3-kb *Bgl*II fragment containing a portion of the putative *PAS7* ORF was used to screen a *P. pastoris* genomic DNA library by filter hybridization (64), and plasmid clones containing overlapping DNA inserts were identified. Restriction mapping of one clone (pSG730) indicated that it contained the remainder of the putative *PAS7* ORF. A 1,766-bp *Spe*I-*Eco*RI fragment containing the entire *PAS7* ORF was found to complement the *pas7-1* mutant. This segment of DNA was sequenced in its entirety on both strands by the chain termination method (65) with a series of gene-specific oligonucleotides as primers.

Plasmids and Southern blot analysis. All manipulations of DNA were performed essentially as described by Sambrook et al. (64). The plasmids pSG700, pSG701, and pSG730 were isolated directly from *P. pastoris* genomic DNA libraries (see above). pSG735 was created by subcloning the 1,766-bp *Spe*I-*Eco*RI fragment from pSG730 between the *Spe*I and *Eco*RI sites of pSL301 (Invitrogen). To create pSG736 (the plasmid used for generating the *pas7*Δ disruption strain SGY130), the ends of the 1.7-kb *Hin*dIII-*Asp*718 fragment of the *P. pastoris LEU2* gene (87) were made blunt by incubation with the Klenow fragment of *E. coli* DNA polymerase I and cloned between the *Eco*47III and *Sal*I sites of pSG735, also made blunt as just described. pSG750 was created by cloning the 717-bp *Eco*RV-*Eco*RI fragment from pSG735 between the *Xmn*I and *Eco*RI sites of pMAL-c2 (New England Biolabs). The MBP-Pas7p protein encoded by this plasmid contains the C-terminal 160 amino acids of Pas7p. pSG751 was created by cloning the 717-bp *Eco*RV-*Eco*RI fragment of pSG735 between the *Pvu*II and *Eco*RI sites of pRSET-C (Invitrogen). The protein encoded by this plasmid also contains the C-terminal 160 amino acids of Pas7p.

A fragment containing 1 kb of DNA immediately upstream of the *PAS7* ORF (including the promoter) was synthesized by PCR with pSG700 as a template and the oligonucleotides 5′-GAAGGGCCCAAGAAGCAATAAACACCAGG-3′ and 5′-GGGCTCGAGATCTAAGATTGGCAGATATGTG-3′ as primers. The PCR product was digested with *Apa*I and *Xho*I and cloned between the *Apa*I and *Xho*I sites in pSG929 (7) to create pSG737. A 1,493-bp fragment containing the *PAS7* ORF and 69 bp of 3′ untranslated DNA was also synthesized by PCR with pSG735 as the template and the oligonucleotides 5′-GAACTCGAGATGC CCCCATCTGAAGAGATCAAG-3′ and 5′-GCCGAATTCCAGAACCAAA CAGAAACCTAAACC-3′ as primers. The PCR product was digested with *Xho*I and *Eco*RI and cloned between the *Xho*I and *Eco*RI sites of pSG737 to create pSG741. This plasmid contained a fragment of the *PAS7* gene extending from 1 kb 5′ of the ORF to 69 bp 3′ of the ORF. The C313S, H315W, C316S, and C318S point mutations were created by PCR with pSG735 as the template and 5′ -CTACTGCAGCTAATTCCGGACATTGCTTCTGTTGGAGCTGC-3′ (C313S), 5′-CTACTGCAGCCAATTGTGGGTGGTGCTTCTGTTGGAGCT GCATAATAG-3′ (H315W), 5′-CTACTGCAGCTAATTGTGGCCATTCCTT CTGTTGGAGCTGCATAATAG-3′ (C316S), and 5′-CTACTGCAGCTAATT GTGGGCATTGCTTCAGCTGGAGCTGCATAATAGACTGG-3′ (C318S), respectively, together with an oligonucleotide that hybridizes near the 3′ end of the gene, 5′-GCCGAATTCCAGAACCAAACAGAAACCTAAACC-3′. Each PCR product was digested with *Pst*I and *Eco*RI, and the 396-bp fragments were cloned between the *Pst*I and *Eco*RI sites of pSG737 to create pSG746, pSG747, pSG748, and pSG749, respectively. The *Pst*I-to-*Eco*RI regions of these clones were sequenced in their entirety to confirm that no unwanted mutations were introduced during PCR. All oligonucleotides were synthesized by Operon Technologies, Inc. DNA was extracted from yeast cells as described previously (36) and used either for recovery of plasmids into *E. coli* or for Southern blot analysis (64).

Antibodies, protein gels, Western blots, and electron microscopy. A bacterial strain carrying pSG750 was induced to synthesize a maltose-binding protein (MBP)-Pas7p fusion protein (containing the C-terminal 160 amino acids of Pas7p). The cells were lysed, and the fusion protein was purified by affinity chromatography on amylose resin as specified by the manufacturer (New England Biolabs). The purified MBP-Pas7p fusion protein was injected into rabbits, and after a series of boost injections, sera was collected. A polyhistidine-Pas7p fusion protein was expressed in the bacterial strain BL21(DE3) (70) carrying pSG751. Anti-Pas7p antibodies raised against MBP-Pas7p were affinity purified against the polyhistidine-Pas7p fusion protein by binding and elution from immobilized polyhistidine-Pas7p (containing the C-terminal 160 amino acids of Pas7p). Affinity-purified anti-Pas4p antibodies were described by Crane et al. (8), and affinity-purified anti-Pas10p antibodies are specific for the C terminus of Pas10p (40). Electrophoresis of proteins was performed on 4 to 20% acrylamide gradient gels as specified by the manufacturer (Bio-Rad), and Western blot (immunoblot) analyses were performed essentially as described by Crane et al. (8). Electron microscopy was performed as described by Gould et al. (32).

Subcellular fractionation, protease protection, and enzyme assays. Cell growth, homogenization, and centrifugation conditions for subcellular fractionations have been described by Crane et al. (8), as have the conditions for protease protection experiments. Catalase assays were performed by the method of Peters et al. (61), and succinate dehydrogenase assays were performed by the method of Pennington (60).

Atomic absorption spectroscopy. MBP-Pas7p fusion proteins were expressed in *E. coli* as described above, except that the cultures were grown in medium containing 1 mM zinc sulfate. Samples were assayed for protein concentration by the method of Bradford (5) with gamma-globulin as a control. Each sample was analyzed for absorption at 214 nm in a Perkin-Elmer flame atomic absorption spectrophotometer and compared with the absorption of standards containing 10 to 100 μM zinc. Zinc-specific absorption was linear within this range, and all the readings of protein samples were between those of the standards. Background zinc values were in the 1 μM range.

RESULTS

Isolation of the *PAS7* gene. Putative clones for the *PAS7* gene were isolated from a *P. pastoris* genomic DNA library by functional complementation of the *pas7-1* mutant. By a combination of nucleotide sequencing, restriction analysis, and complementation analysis, a single 1,257-bp ORF that complemented the *pas7-1* mutant was identified (Fig. 1). Most of the

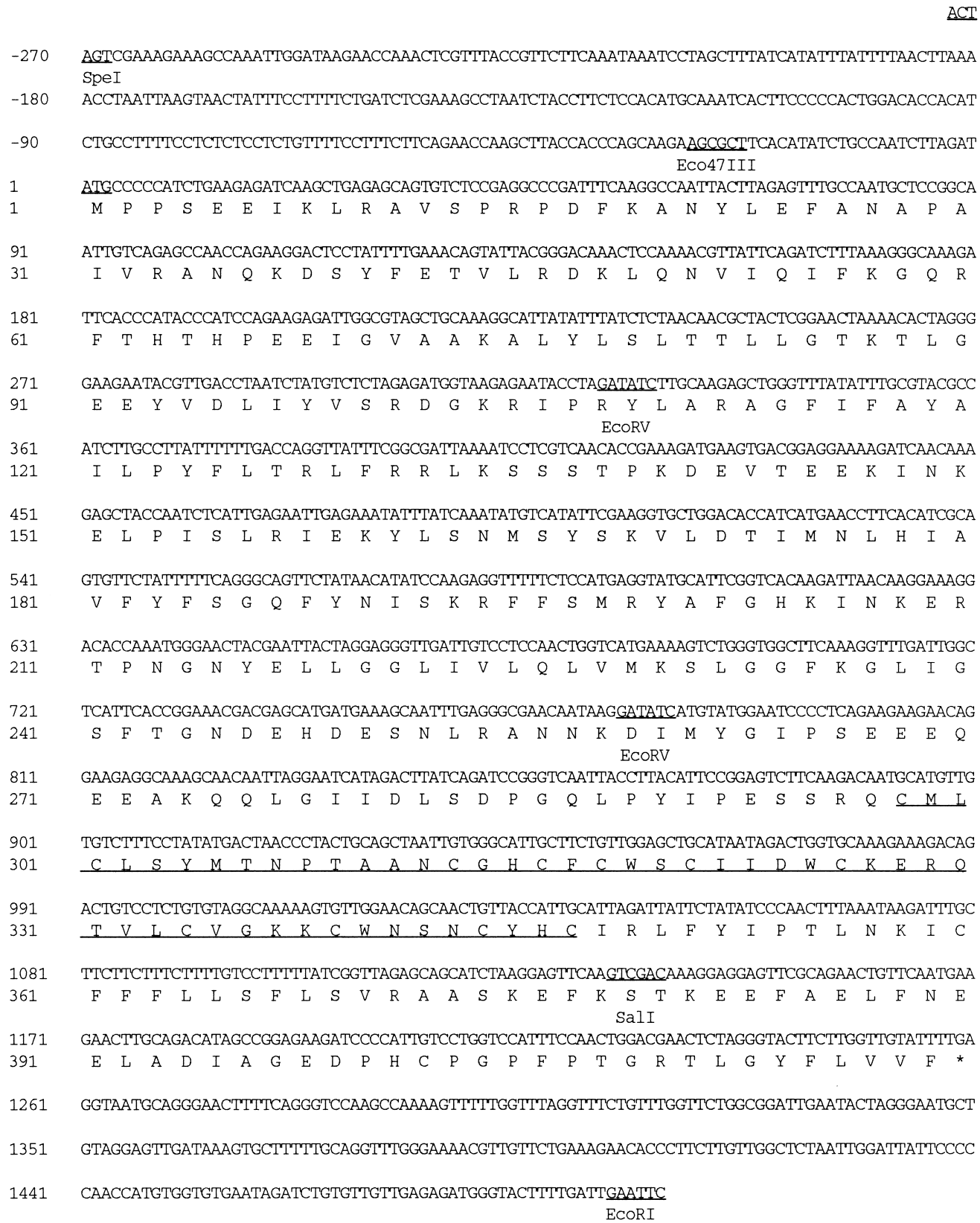

FIG. 1. Nucleotide sequence of the *PAS7* gene and deduced amino acid sequence of Pas7p. The start codon is underlined, and the stop codon is marked by an asterisk. The *Spe*I, *Eco*47III, *Eco*RV, *Sal*I, and *Eco*RI sites are underlined and labeled. The amino acid sequence corresponding to the C_3HC_4 domain of the protein is also underlined. The nucleotide sequence was determined in its entirety on both strands.

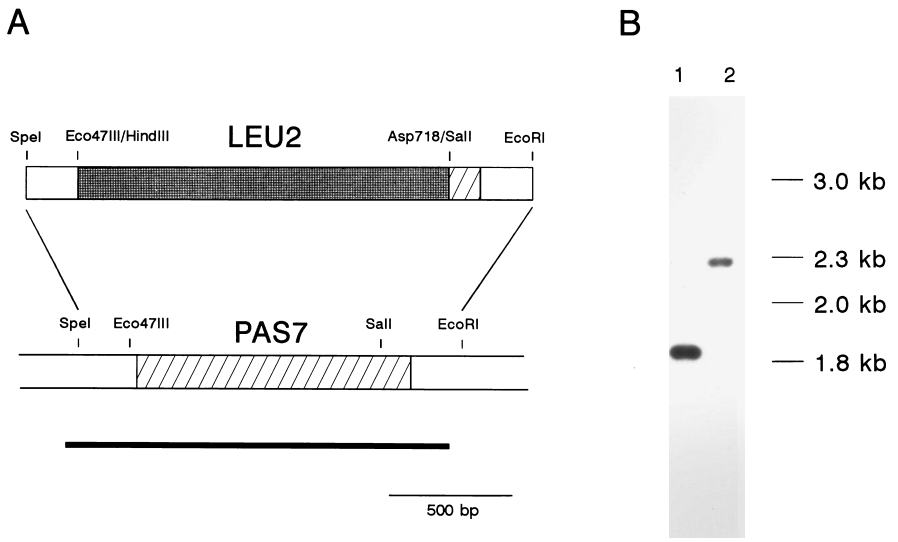

FIG. 2. Disruption of the *PAS7* gene. (A) Diagram of the 2.3-kb *Spe*I-*Eco*RI fragment of pSG936 (top), the wild-type *PAS7* locus (middle), and the *Spe*I-*Eco*RI fragment used as probe in the Southern blot experiments (solid line underneath). The 2.3-kb *Spe*I-*Eco*RI fragment from pSG936 was transfected into the *leu2*Δ strain SGY53 (87), and LEU2$^+$ transformants were selected. One of these was designated SGY130. Homologous recombination between the introduced DNA fragment and the *PAS7* locus would delete almost the entire *PAS7* ORF, integrate the *LEU2* gene in its place, and increase the size of the *Spe*I-*Eco*RI fragment from 1.8 to 2.3 kb. (B) The LEU$^+$ transformant SGY130 was screened by Southern blotting to determine if it carried the desired disruption of the *PAS7* gene. Total DNA was prepared from SGY130 and SGY53, the parental *PAS7* strain. After digestion with *Spe*I and *Eco*RI, these samples were analyzed by Southern blotting with the 1.8-kb *Spe*I-*Eco*RI fragment from *PAS7* as the probe. As expected, SGY53 contained a 1.8-kb hybridizing fragment (lane 1). In contrast, SGY130 contained a 2.3-kb hybridizing band (lane 2) and had undergone the desired gene disruption event. The lower intensity of the band in lane 2 reflects the loss of 1.2 kb of *PAS7* DNA in this strain.

chromosomal copy of the complementing gene was deleted by one-step gene disruption (63), creating strain SGY130 (Fig. 2). This strain was unable to grow on methanol or fatty acids, phenotypes identical to those of the original *pas7-1* mutant. When the *pas7-1* strain PPY26 was crossed with SGY131 (the disruption mutant in a *his4*Δ background), the resultant diploid cells were unable to grow on methanol, indicating that the two mutations were in the same complementation group. These diploids were then sporulated, and the meiotic products were analyzed. Of the over 200 tetrads examined, none contained PAS$^+$ spores, indicating that the disruption mutation was allelic to *pas7-1*. The above tests demonstrated that the gene we cloned was the authentic *PAS7* gene and not a suppressor gene. Hereafter, the disruption mutant is referred to as the *pas7*Δ mutant.

Pas7p contains a C_3HC_4 zinc-binding domain essential for activity. Examination of the 419-amino-acid deduced sequence of Pas7p revealed that it contained a cysteine-rich region near its carboxy terminus (amino acids 298 to 347) that matched the consensus sequence of the C_3HC_4 putative zinc-binding motif (17) (Fig. 3). To address the possibility that zinc binding is important for the function of Pas7p, we created point mutations within the C_3HC_4 domain of the *PAS7* gene. Two cysteine residues of the C_3HC_4 motif were changed to serine (C313S and C318S), the conserved histidine was changed to tryptophan (H315W), and an additional cysteine that was not part of the C_3HC_4 motif was also changed to serine (C316S). Neither serine nor tryptophan is able to participate in coordination of zinc ions. Each mutant *PAS7* gene was cloned into a yeast replicating vector and examined for *PAS7* activity, assayed here by testing the ability of each mutant to complement the methanol growth defect of a *pas7*Δ strain. Strain SGY131 (*pas7*Δ *his4*Δ) was transformed with five different *HIS4*-based replicating plasmids: pSG741, carrying the wild-type *PAS7* gene; pSG746, carrying the C313S mutant; pSG747, carrying the H315W mutant; pSG748, carrying the C316S mutant; and pSG749, carrying the C318S mutant. The resultant strains were numbered on the basis of the plasmid they contain (e.g., SGY747 is strain SGY131 carrying plasmid pSG747). As expected for a strain carrying the wild-type *PAS7* gene, SGY741 grew well on methanol (Fig. 3). In contrast, SGY746, SGY747, and SGY749 were not able to proliferate on methanol, indicating that the C313S, H315W, and C318S mutations eliminated *PAS7* activity. Although cysteine 316 was no more than 4 amino acids away from the essential Cys-313, His-315, and Cys-318 residues, it was not a conserved residue of the C_3HC_4 motif. The strain carrying the C316S mutant (SGY748) grew as well as the strain carrying the wild-type *PAS7* gene (SGY741), demonstrating that Cys-316 was not essential for *PAS7* function.

The C_3HC_4 domain of Pas7p binds zinc. Because the above data are consistent with a role for zinc binding in Pas7p function, the ability of Pas7p to bind zinc via its C_3HC_4 domain was examined. An MBP-Pas7p fusion protein (containing the C-terminal 160 amino acids of Pas7p, including the C_3HC_4 domain) was expressed in *E. coli*, purified, and examined by atomic absorption spectrometry. The zinc and protein concentrations of this sample were approximately 46 and 35 μM, respectively. This zinc/protein ratio of 1.3:1 is close to the value of 2 zinc ions per C_3HC_4 domain predicted from the three-dimensional structure of the C_3HC_4 domain in the Vmw110 protein (2). To test if zinc was tightly bound to purified MBP-Pas7p, the sample was dialyzed overnight against 1 mM EDTA and again analyzed by atomic absorption spectrometry. Following this treatment, the ratio of zinc to protein in the sample was unchanged. As a control, a second MBP fusion protein devoid of Pas7p sequences (MBP-Pxr1p [12]) was analyzed by atomic absorption spectrometry. Even though the concentration of MBP-Pxr1p (75 μM) was more than twice that of MBP-Pas7p, no zinc was detected in this fusion protein. The C313S mutant version of the Pas7p C_3HC_4 domain (MBP-Pas7p/C313S) was also synthesized and examined by atomic absorption spectrometry. It contained roughly half the amount

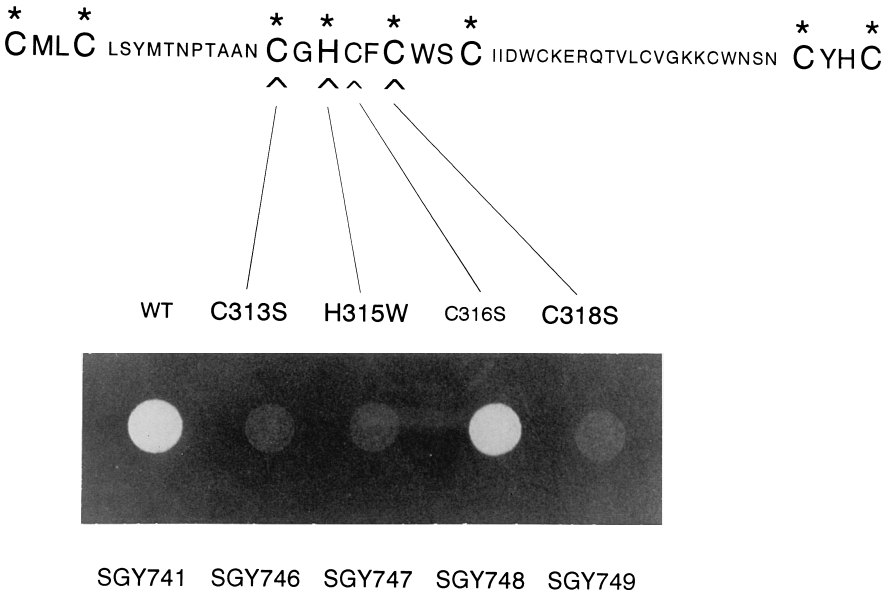

FIG. 3. Effect of point mutations on *PAS7* activity. The 50 amino acids that make up the C_3HC_4 domain of Pas7p are shown at the top of the figure, and the eight amino acids which are thought to coordinate the zinc ions are highlighted by asterisks. The *pas7*Δ strains carrying wild-type (WT) or mutant *PAS7* genes were spotted onto methanol minimal medium. After a 4-day incubation, the plate was photographed.

of zinc (40 μM zinc and 54 μM protein, a ratio of 0.7:1) as the wild-type Pas7p C_3HC_4 domain did. A similar analysis of an MBP-Pas7p/C316S fusion protein revealed that the C316S mutation did not substantially alter the zinc-binding characteristics of the protein (78 μM zinc and 50 μM protein, a ratio of 1.5:1).

***PAS7* is required for import of peroxisomal matrix proteins.** In *P. pastoris*, loss of the PTS1 receptor (Pas8p) leads to an inability to import PTS1 proteins into peroxisomes but does not affect the import of PTS2-containing proteins such as thiolase (52). Since catalase is not imported into peroxisomes of the *pas8* mutant whereas thiolase is imported (52), *P. pastoris* catalase may be considered a marker for PTS1-mediated protein import whereas thiolase appears to be a good marker for PTS2-mediated import. Wild-type and *pas7*Δ cells were incubated in oleic acid medium, spheroplasted, homogenized, and separated into 25,000 × *g* supernatant (S) and pellet (P) fractions. The P fraction contains mostly peroxisomes and mitochondria, while the S fraction contains cytoplasm and small organelles (8). Equal proportions of S and P were assayed for catalase activity and thiolase protein (Fig. 4). In wild-type cells, significant proportions of both catalase (46% of the total activity) and thiolase (75% of the total) were associated with the organelle pellet. In contrast, less than 1% of the catalase activity and less than 5% of the thiolase were associated with the organelle pellet in the *pas7*Δ cells (the thiolase immunoblot was quantitated by densitometry). Thus, loss of Pas7p resulted in a defect in the import of both catalase and thiolase into peroxisomes.

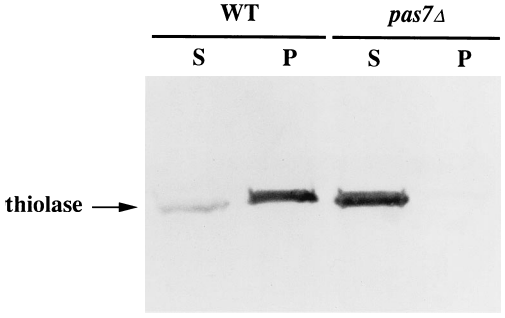

FIG. 4. Distribution of thiolase in wild-type (WT) and *pas7*Δ cells. Equal proportions of organelle pellet (P) and cytoplasmic supernatant (S) fractions obtained from strains SGY55 (WT) and SGY131 (*pas7*Δ) were separated by SDS-PAGE and immunoblotted with affinity-purified anti-thiolase antibodies.

Studies on several other *P. pastoris pas* mutants (*pas1*, *pas4*, and *pas5*) have shown that while each is defective in import of both PTS1 and PTS2 proteins, each is capable of importing a small amount of peroxisomal matrix proteins into peroxisomes of normal density (35, 39, 69). In the human equivalents of yeast *pas* mutants, i.e., cells from patients with PBD, a similar import ability has been found for PBD cell lines representing most complementation groups. However, no import of peroxisomal matrix proteins was detected in PBD cell lines representing two complementation groups (CG3 and CG10). Thus, there appear to be two classes of mutants defective in import of both PTS1 and PTS2 proteins: translocation competent and translocation defective. To determine whether loss of Pas7p had a complete or partial matrix protein import defect, we examined the distribution of a peroxisomal matrix protein in the *pas7*Δ mutant more closely. Dense organelle pellets obtained from wild-type, *pas4*Δ, and *pas7*Δ cells were each separated by sucrose density gradient centrifugation. Fractions were collected from the bottom of the gradients and assayed for catalase activity (peroxisomal marker enzyme), succinate dehydrogenase activity (mitochondrial marker enzyme), and density. Whereas a gradient profile of wild-type cells shows a large peak of catalase at a density of 1.22 g/cm^3 and a gradient profile from *pas4*Δ cells exhibits a small peak of catalase at the same density, *pas7*Δ cells contain only a single peak of catalase activity at the top of the gradient (Fig. 5). The inability of the *pas7*Δ mutant to import this peroxisomal matrix protein into vesicles with the normal density of peroxisomes indicated that unlike the *pas1*, *pas4*, *pas5*, and *pas8* mutants (35, 39, 52, 69),

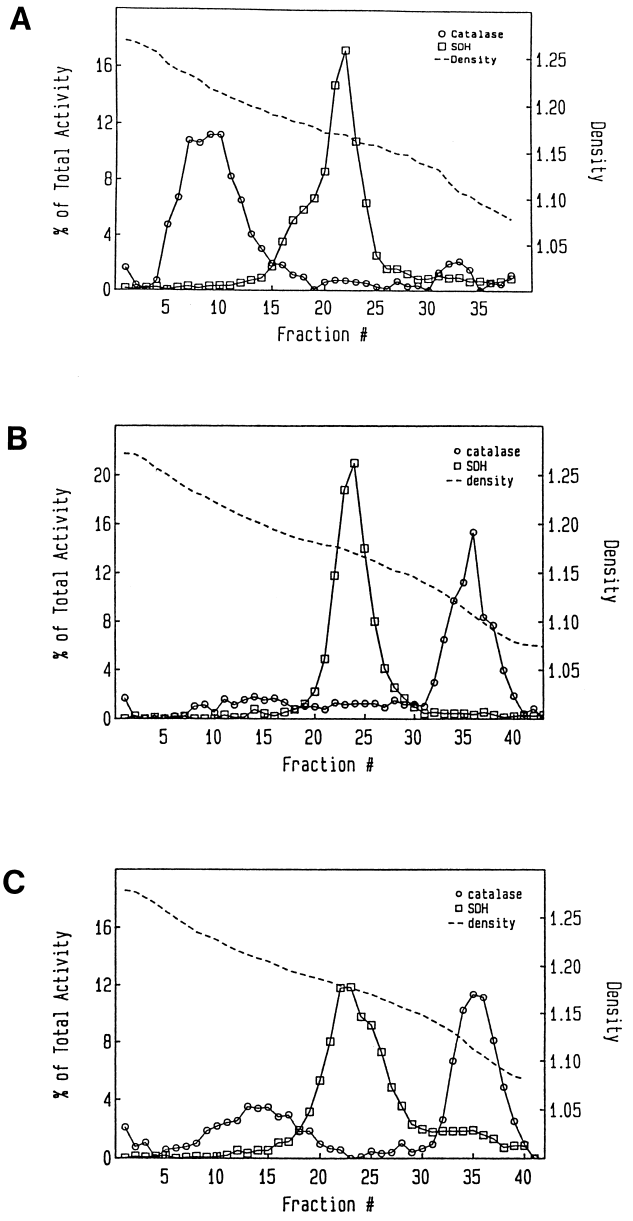

FIG. 5. The *pas7*Δ mutant is unable to import catalase into peroxisomes of normal density. Wild-type, *pas7*Δ, and *pas4*Δ cells were incubated in oleic acid medium and homogenized, and 25,000 × *g* organelle pellets were isolated. Each pellet was resuspended and fractionated by sucrose density centrifugation (8). The relative catalase activities of the different fractions are represented by open circles, and the relative succinate dehydrogenase activities are represented by open boxes. Enzyme profiles from SGY55, a wild-type strain (A), SGY131, a *pas7*Δ strain (B), and SGY101, a *pas4*Δ strain (C) are shown.

the *pas7*Δ mutant was completely defective in matrix protein import.

Morphology of peroxisomal intermediates in the *pas7*Δ mutant. Wild-type and *pas7*Δ cells were incubated in medium containing either methanol or oleic acid and examined by transmission electron microscopy. As reported previously for *P. pastoris* (32, 35, 40, 52, 69), wild-type cells incubated in methanol medium contained clusters of large peroxisomes (Fig. 6A). In contrast, such structures were never observed in *pas7*Δ cells incubated in methanol medium. Instead, we observed unusual sheets of membrane, usually two to six layers per stack (Fig. 6B to F). These structures were detected in 41% of the cell profiles examined and in almost all instances were found within invaginations of the vacuole (Fig. 6B to D). In those sections in which the structures were not within an invagination of the vacuole (only 2%), they were usually located at the surface of the vacuole (Fig. 6E) but were occasionally observed in the cytoplasm (Fig. 6F). Even when seemingly free in the cytoplasm, these structures resembled sheets rather than vesicles (Fig. 6E). The fact that some of these structures contained electron-dense globular structures indistinguishable from ribosomes (Fig. 6F) indicates that they may simply be curved membrane sheets with cytoplasmic components in the space defined by their concave surface.

We also examined the morphology of wild-type and *pas7*Δ cells incubated in oleate medium. Wild-type *P. pastoris* incubated in oleic acid medium accumulates numerous small, detached peroxisomes (Fig. 7A) as reported previously (32, 35, 40, 52, 69). The *pas7*Δ cells incubated in oleic acid-containing medium did not contain peroxisomes with normal morphology but did contain unusual membranous structures in invaginations of the vacuole (Fig. 7B to D). These structures were detected in 59% of the cell profiles examined, found only within vacuolar invaginations, and differed from those observed in the methanol-induced *pas7*Δ cells (Fig. 7B to D) in that (i) they were so small that they usually appeared as dots rather than sheets; (ii) they were more abundant than the sheets observed in methanol-induced *pas7*Δ cells; and (iii) when they did appear as sheets, they were much shorter than those observed in methanol-induced *pas7*Δ cells. These differences are quite similar to those documented for peroxisomes of wild-type cells: peroxisomes in cells grown on methanol are larger, clustered, and less numerous than those from cells grown in oleate medium (compare Fig. 6A and 7A) (32, 35, 40, 52, 69).

Association between peroxisomes and vacuoles in wild-type cells. Because membrane sheets were observed within vacuolar invaginations so frequently in *pas7*Δ cells, we reexamined the relative distribution of peroxisomes and vacuoles in wild-type cells incubated in methanol medium. An apposition of the peroxisome and vacuole was detected in 97% (203 of 210) of the observed profiles. Furthermore, peroxisomes could be detected in invaginations of the vacuole (Fig. 8A and B) and sometimes appeared to form membranous connections to the vacuole (Fig. 8C and D). The close association between peroxisomes and vacuoles can also be seen in previously published electron micrographs of wild-type *P. pastoris* (32, 35, 40, 52, 69), as well as between peroxisome ghosts of the *pas1* mutant and the vacuole (35). Interestingly, we occasionally observed one or more membrane sheets within peroxisome-containing invaginations of the vacuole in wild-type cells (Fig. 8F). Although they resemble the intermediates observed in the *pas7*Δ cells, these sheets did not appear in large clusters and sometimes appeared to contain a lumen space near their termini. Peroxisomes of wild-type cells grown in oleic acid medium were also observed in apposition to vacuoles, although much less frequently (only 20% of the sections) than in methanol-grown cells.

Pas7p is an integral peroxisomal membrane protein. The subcellular distribution of Pas7p was determined by using affinity-purified antibodies generated against the carboxy-terminal 160 amino acids of Pas7p. These antibodies detected a single protein with an apparent molecular mass of 48 kDa in whole-cell extracts from wild-type cells but not in the *pas7*Δ strain SGY130 (data not shown). This relative molecular mass (48 kDa) is consistent with the size predicted from the deduced amino acid sequence of Pas7p. Further analysis revealed that

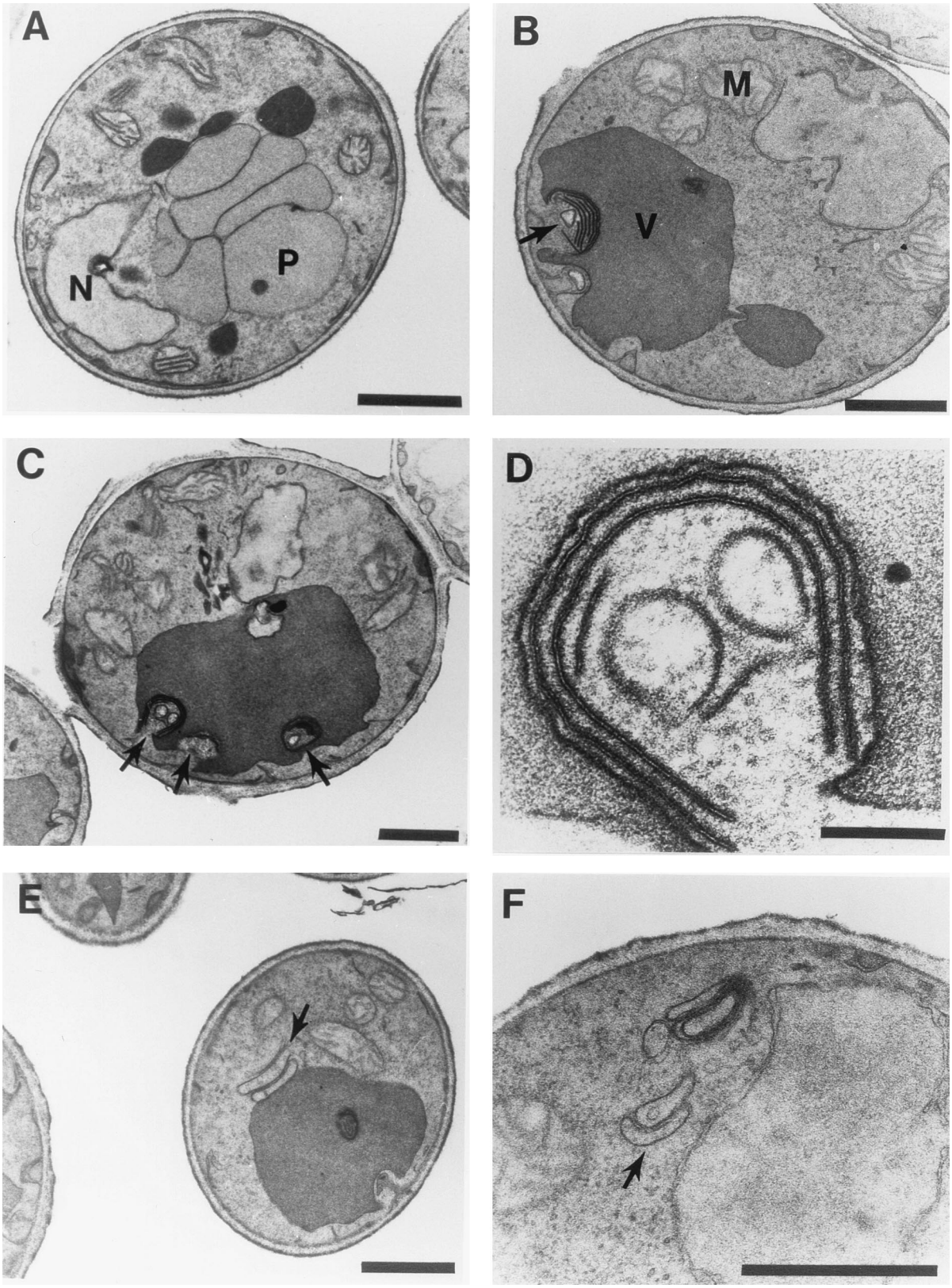

FIG. 6. Electron microscopic analysis of wild-type and *pas7*Δ cells incubated in methanol medium. (A) SGY55, a wild-type cell. (B to F) SGY131, *pas7*Δ cells. The arrows point to the membrane sheets that accumulate in the *pas7*Δ mutant. A high magnification of the membrane sheets in an invagination of the vacuole shown in panel C is presented in panel D. Note the double-membrane appearance of the parallel sheets. P, peroxisome; N, nucleus; M, mitochondria; V, vacuole. Bars, 1 μm.

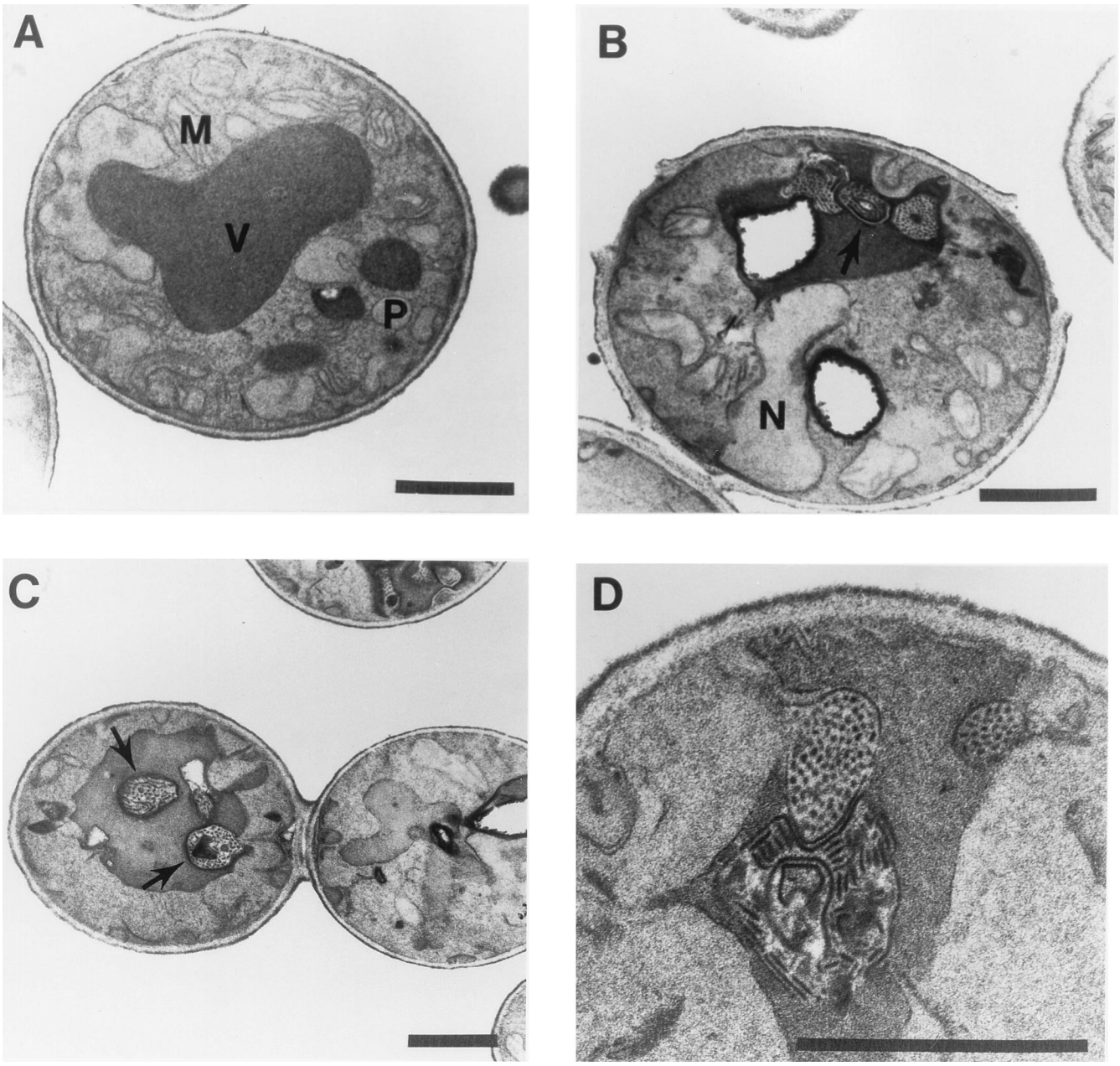

FIG. 7. Electron-microscopic analysis of wild-type and *pas7*Δ cells incubated in oleic acid medium. (A) SGY55, a wild-type cell. (B to D) SGY131, *pas7*Δ cells. Arrows point to the membranous structures in invaginations of the vacuole. Note that the membranous sheets, when present, are much shorter than those detected in the methanol-induced cells. P, peroxisome; N, nucleus; M, mitochondria; V, vacuole. Bars, 1 μm.

Pas7p was most abundant during growth on fatty acids and methanol but was barely detectable in glucose-grown cells (data not shown).

Wild-type cells grown in fatty acid medium were converted to spheroplasts, homogenized, and separated by differential centrifugation into two fractions: a pellet that contained primarily mitochondria and peroxisomes and a supernatant that contained cytosol and light organelles. Equivalent amounts of each fraction were assayed by Western blot. Pas7p was detected exclusively in the organelle fraction (Fig. 9). To determine the subcellular distribution of Pas7p more precisely, the organelle pellet was fractionated by sucrose density gradient centrifugation. Fractions were collected and assayed for catalase activity (a peroxisomal marker enzyme), succinate dehydrogenase activity (a mitochondrial marker enzyme), and density. Both peroxisomes and mitochondria were found at their expected densities (1.22 and 1.17 g/cm^3, respectively). Western blot analysis of these fractions revealed that Pas7p was found exclusively in the peroxisomal fractions (Fig. 10).

Although Pas7p was clearly a peroxisomal protein, it remained to be determined whether it was located in the matrix of the peroxisome or was a component of the organelle membrane. An organelle fraction was lysed under hypotonic conditions and separated by centrifugation into a supernatant containing matrix proteins and a pellet enriched for membrane proteins. Pas7p was detected only in the pellet, indicat-

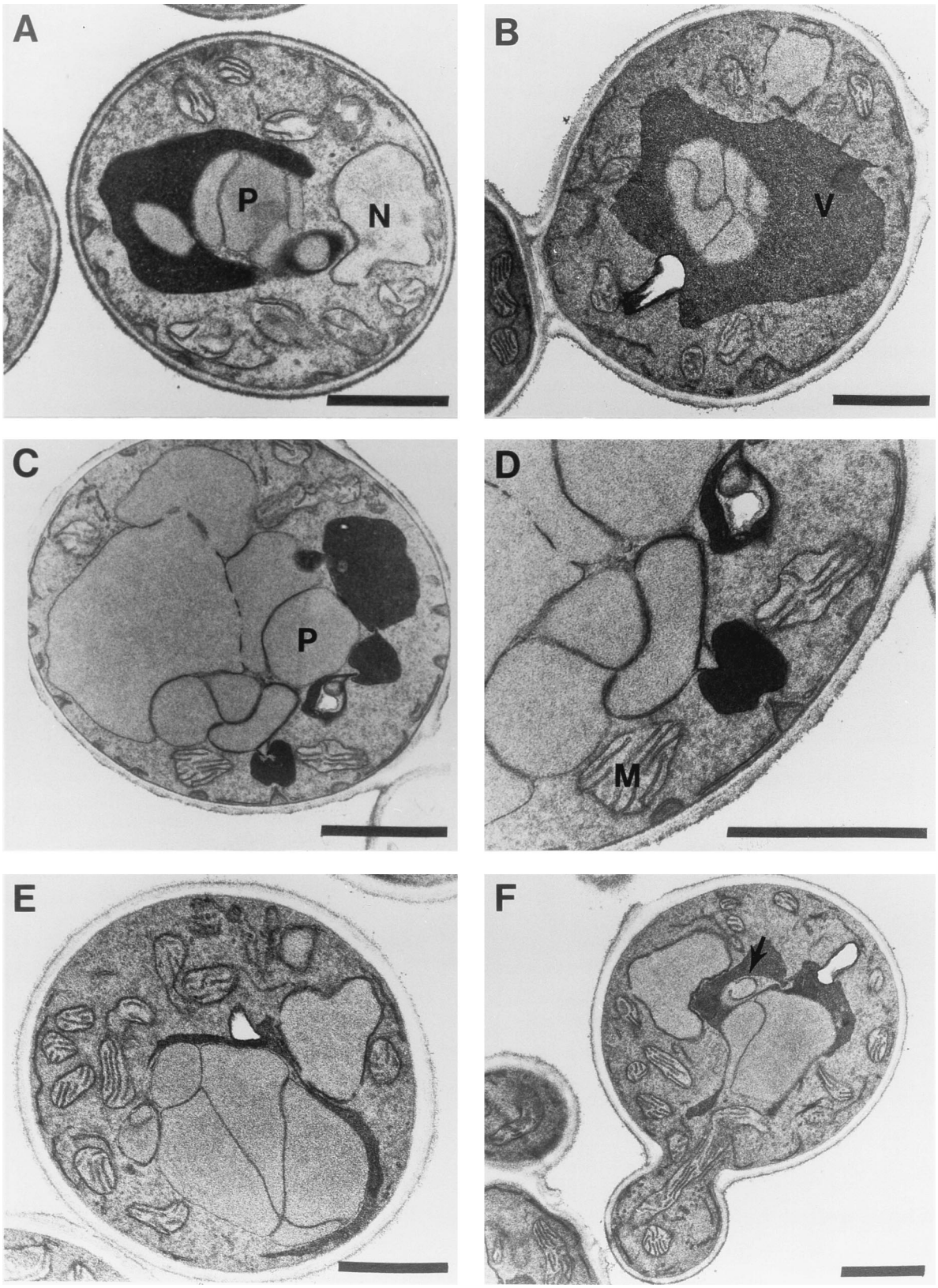

FIG. 8. Peroxisomes in wild-type *P. pastoris* grown in methanol medium are found in close association with vacuoles. In all panels, peroxisomes in SGY55, a wild-type strain, were almost always found in contact with the vacuole. (A and B) Sections demonstrating the presence of peroxisomes in invaginations of the vacuole, even during log-phase growth in methanol medium, were common. (C and D) Vacuoles were often found at the periphery of peroxisomes and in some instances appeared to form membranous extensions which contacted the peroxisome. Interestingly, membrane sheets (arrow in panel F), which may represent newly synthesized peroxisomes, could sometimes be observed in invaginations of the vacuole (arrow in panel F). P, peroxisome; N, nucleus; M, mitochondria; V, vacuole. Bars, 1 μm.

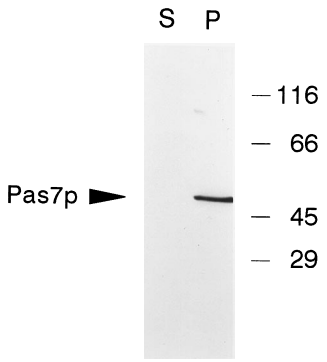

FIG. 9. Organelle association of Pas7p. Wild-type cells were homogenized and separated by differential centrifugation into a supernatant containing cytosol and light organelles and a pellet containing primarily mitochondria and peroxisomes. Equal amounts of each fraction were separated by SDS-PAGE and analyzed by Western blot with affinity-purified anti-Pas7p antibodies.

ing that it was a membrane protein (data not shown). To assess whether Pas7p was an integral or peripheral peroxisomal membrane protein, an organelle membrane preparation was resuspended and homogenized in 100 mM Na_2CO_3 (pH 11.5), a treatment that strips all but integral proteins from membranes (18, 19). After a 30-min incubation, the sample was spun at 200,000 $\times$ *g* for 30 min, yielding a supernatant containing matrix and peripheral membrane proteins and a pellet containing membrane lipids and integral membrane proteins. Pas7p remained membrane associated after this treatment, suggesting that it was an integral peroxisomal membrane protein (Fig. 11). In contrast, Pas4p, a peripheral peroxisomal membrane protein (8), was extracted completely from membranes by this treatment. Consistent with these results, hydropathy analysis (43) of Pas7p revealed that it contained two hydrophobic segments (amino acids 173 to 192 and 218 to 236) long enough to span a biological membrane (data not shown).

Pas7p lies within the peroxisome. The anti-Pas7p antibodies

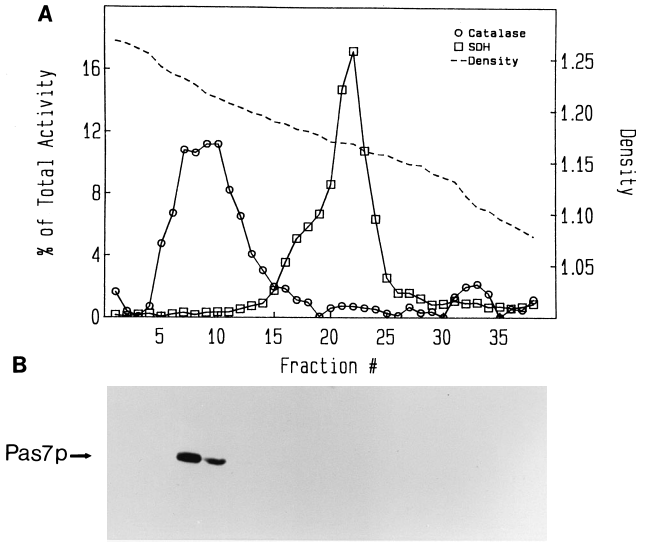

FIG. 10. Subcellular localization of Pas7p. An organelle pellet enriched for peroxisomes and mitochondria was separated by sucrose density gradient centrifugation. Each fraction from the gradient was assayed for catalase activity, succinate dehydrogenase (SDH) activity, and density. (A) Enzyme activities are presented as a percentage of the total activity of all fractions combined. (B) Every third fraction (1, 4, 7, etc.) was assayed for Pas7p by Western blotting.

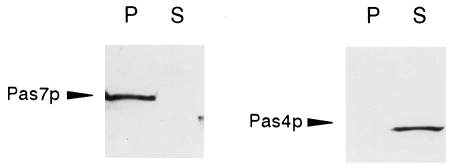

FIG. 11. Carbonate extraction fails to release Pas7p from the membrane. An organelle pellet was hypotonically lysed, and the membranes were resuspended in 100 mM Na_2CO_3 (pH 11.5) at a final protein concentration of 0.7 mg/ml (18, 19). After a 30-min incubation on ice, the sample was centrifuged at 200,000 $\times$ *g* for 30 min. Equal amounts of the resultant supernatant and pellet were analyzed by Western blotting for Pas7p as well as for Pas4p, a known peripheral peroxisomal membrane protein (5).

(specific for amino acids 259 to 419 of Pas7p) were next used to examine the orientation of Pas7p in the membrane. An organelle pellet fraction was resuspended and incubated with different amounts of the protease trypsin in the presence or absence of the detergent Triton X-100. Following the incubations, equal proportions of each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and examined by Western blot with antibodies specific for Pas7p, thiolase, and Pas10p (Fig. 12). Both Pas7p and thiolase (a known peroxisomal matrix protein) were resistant to trypsin in the absence of detergent but were degraded when the peroxisome membrane was disrupted with detergent. In contrast, Pas10p, an integral peroxisomal membrane protein which faces the cytoplasm (40), was completely degraded by exogenous trypsin regardless of the integrity of the peroxisomal membrane. Thus, these data suggested that Pas7p is an integral peroxisomal membrane protein with its hydrophilic segments extending into the peroxisomal lumen. Furthermore, because the 50 trypsin sites in Pas7p are distributed throughout the entire length of the protein with no two sites further than 31 amino acids apart, it is unlikely that any extended segment of the protein exists outside of the peroxisome.

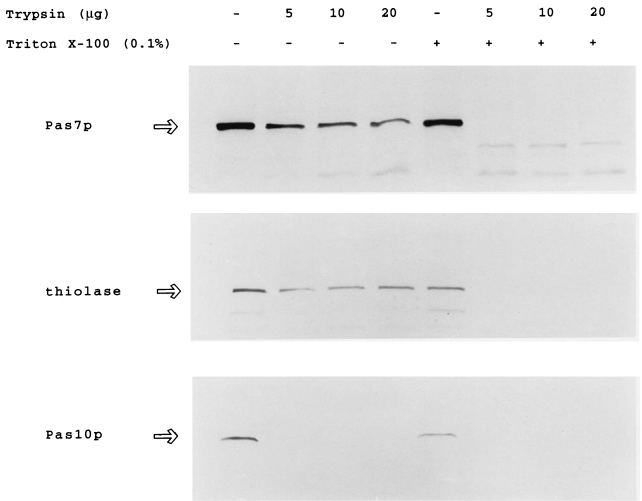

FIG. 12. Pas7p is protected from proteolysis by the peroxisome membrane. Cellular organelles (200-μg protein equivalents) were incubated with 5, 10, and 20 μg of trypsin in the presence or absence of 0.1% Triton X-100. These samples were then separated by SDS-PAGE and immunoblotted with antibodies specific for Pas7p, thiolase, and Pas10p.

DISCUSSION

Phenotype of the *pas7*Δ mutant. Peroxisome assembly mutants may be subdivided into three basic groups: those specifically unable to import PTS1 proteins, those specifically unable to import PTS2 proteins, and those with a defect in import of both PTS1 and PTS2 proteins. Complementation analyses of both yeast *pas* mutants and human cells from PBD patients have shown that the PTS1-specific (type 1) defect is caused by mutation in one gene, the PTS2-specific (type 2) defect is caused by mutation in another, and the defect in both PTS1 and PTS2 protein import (type 3) results from loss of any of the remaining 13 or more peroxisome assembly genes (13, 55, 68). In addition, Slawecki et al. (68) reported that the type 3 defect in human PBD cell lines can be either severe, with no detectable import of matrix proteins, or partial, with some import of PTS1 and PTS2 proteins. Interestingly, the severe type 3 defect is relatively rare, occurring in only 15% of the type 3 cell lines. Thus, of human cells defective in import of both PTS1 and PTS2 proteins, most (85%) retain the ability to translocate some peroxisomal matrix proteins into the organelle. The analysis of yeast *pas* mutants is starting to yield similar results. The *pas1*, *pas4*, and *pas5 P. pastoris* mutants have defects in both PTS1 and PTS2 import but are able to translocate small amounts of matrix proteins into peroxisomal intermediates (35, 39, 69). In contrast, we found that the *P. pastoris pas7*Δ mutant exhibits a more severe type 3 import defect since it is unable to import matrix proteins into peroxisomes of normal density. Nevertheless, the *pas7*Δ mutant does appear to contain peroxisomal intermediates. Electron micrographs of *pas7*Δ cells incubated in peroxisome-inducing media showed the presence of membranous sheets and minivesicles which appeared to lack lumen space. These structures were abundant only after incubation in peroxisome-inducing medium (methanol or oleic acid). Furthermore, the morphology of these structures varied with carbon source in the same way that the morphology of peroxisomes in methanol-induced cells differs from that of peroxisomes in fatty acid-induced cells: larger, fewer, attached structures upon methanol induction, and smaller, more numerous, detached structures upon oleate induction. Thus, these structures appeared to be peroxisomal intermediates which accumulate in the absence of Pas7p.

In addition, these structures were often found in close association with the vacuole. We and others have shown that this is a common feature of peroxisomes from *P. pastoris* (32, 35, 40, 52, 69). It has been proposed that vacuoles may degrade peroxisomes by engulfment after a switch from peroxisome-requiring environmental conditions to high-glucose medium (79). However, in our experiments, the cells were in log-phase growth on peroxisome-requiring media when harvested for the electron microscopy experiments. One possibility is that vacuoles maintain a close association with peroxisomes so that when the environmental conditions no longer demand the activity of peroxisomal enzymes, the cell can rapidly convert their amino acids into other macromolecules. There would be a strong selective advantage for such a mechanism, because the peroxisomal enzymes alcohol oxidase and dihydroxyacetone synthase make up more than 50% of the total cell protein when *P. pastoris* is grown on methanol.

Potential roles for Pas7p in peroxisome assembly. Prior studies on peroxisome-deficient human cells have shown that loss of peroxisome assembly factors can result in formation of peroxisome ghosts, membrane-enclosed structures with considerable internal volume (66). Structures similar to ghosts with obvious lumen space have also been detected in the *P. pastoris pas1*, *pas4*, *pas5*, *pas8*, and *pas10* mutants (35, 39, 40, 52, 69). In contrast, the electron microscopy data indicated that peroxisomal intermediates in *pas7*Δ cells lack lumen space. Thus, Pas7p must be required both for elaboration of the peroxisome lumen and for entry of peroxisomal matrix proteins and would be expected to play a direct role in at least one of these two processes.

The most compelling evidence in support of a role for Pas7p in protein translocation is the inability of *pas7*Δ to cells to import PTS1 and PTS2 proteins into peroxisomes of normal density. In addition, Pas7p has the appropriate subcellular and subperoxisomal location for such a role, since it is an integral peroxisomal membrane protein. In fact, of the 11 known peroxisome assembly factors, Pas7p is the only integral peroxisomal membrane protein known to reside entirely within the peroxisome (35, 37, 39, 40, 50, 52, 69, 83). The hypothesis that Pas7p is a protein translocation factor also implies that entry of most lumen contents must be either directly or indirectly dependent upon protein translocation across the membrane. For example, if one assumes that Pas7p is directly involved only in protein translocation across the peroxisome membrane and that water and ions are normal components of the peroxisome lumen, the absence of luminal space in the *pas7*Δ mutant implies that water and ions cannot enter the peroxisome without a functional protein translocation apparatus. While speculative, the possibility that protein translocation is required for water and ion transport into the peroxisome is supported by the recent observations of Glover et al. (24) and McNew and Goodman (53). In these studies, the authors demonstrated that protein oligomers can be imported into peroxisomes. Because it would not be possible to translocate proteins in the folded, oligomeric state across the peroxisome membrane without also importing the water molecules and ions bound to their surface, entry of proteins in a folded state would appear to require cotransport of ions and water along with the proteins. The absence of luminal space within the peroxisomal intermediates of the *pas7*Δ mutant also implies that substrate transport into the peroxisome may be eliminated by loss of Pas7p. If transport of substrates from the cytoplasm to the peroxisome is sensitive to the metabolic activities within the organelle, a cessation of substrate transport by membrane transporters would, in fact, be the expected result in the absence of matrix enzyme activity.

While the phenotypes of the *pas7*Δ mutant may all be explained by a protein translocation defect, it is also conceivable that *pas7*Δ plays a direct role in formation of the peroxisome lumen rather than in protein translocation. Formation of a requisite protein-lipid environment in the peroxisome membrane or creation of a pore that allows entry of another necessary component are both reasonable possibilities. For example, Pas7p may be required for activation of other peroxisomal membrane proteins, including channels, transporters, and pores. Also, some other molecule might have to enter the peroxisome prior to elaboration of the lumen and Pas7p might be part of a channel that allows entry of such a factor.

Pas7p is not required for peroxisome proliferation. The *H. polymorpha* ortholog of Pas7p, HpPer8p, has recently been described by Tan et al. (74) (Fig. 13). In contrast to our findings and interpretations regarding the roles of Pas7p in peroxisome assembly, Tan et al. (74) have proposed that HpPer8p is a component of the molecular machinery that controls peroxisome proliferation. This is based largely on their observation that overexpression of HpPer8p leads to increased numbers of peroxisomes. Although this result is consistent with a possible role for HpPer8p in peroxisome proliferation, it is also the result expected if Pas7p/HpPer8p simply performed the rate-limiting step in peroxisome assembly. Because the peroxisomal intermediates of the *pas7*Δ mutant proliferate in response to

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PpPas7p         MPPSEEIKLRAVSPRPDFKANYLEFANAPAIVRANQKDSYFETVLRDKLQNVIQIFKGQR   60
                          ||    | ||||||||||||||||||: |:: | :|:  :|| :
HpPer8p                  MFK..LLSFANAPAIVRANQKDSYFESRLHNQLLDVVKAIKGSH   42

PpPas7p         FTHTHPEEIGVAAKALYLSLTTLLGTKTLGEEYVDLIYVSRDGKRIPRYLARAGFIFAYA  120
                | |  |||:   | |||| ||||:|:||||||||||:||||||::||:: :| ||:||
HpPer8p         FVHKYPEELRTLATALYLCLTTLVGSKTLGEEYVDLVYVSRDGRKIPKFASRFGFVVAYV  102

PpPas7p         ILPYFLTRLFRRLKSSSTPKDEVTEEKINKELPISLRIEKYLSNMSYSKVLDTIMNLHIA  180
                ::|| :  |: :||:  :                  |:  ::| :||  |:| ::|||:|
HpPer8p         LFPYAVRQLLQKLKAQQS.................RLAQLVSGVSYMNVMD.LLNLHLA  143

PpPas7p         VFYFSGQFYNISKRFFSMRYAFGHKINKER.TPNGNYELLGGLIVLQLVMKSLGGFKGLI  239
                :|||:| :|: :||:|::|||| :::|:   : ||||||| ||::| |:| :: :: |
HpPer8p         LFYFTGKYYQFAKRLFGLRYAFGYRVDKNQQRARGNYELLGLLIIFQTVFKNVANLRKLW  203

PpPas7p         GSFTGNDEHDESNLRANNKDIMYGIPSEEEQEEAKQQLGIIDLSDPGQLPYIPESSRQCM  299
                |  :     | ::|       :| :      ::: :   :|||:||  |||:||:|| ||
HpPer8p         G..ATKTVQDSGDL.......IYRF.....RDQTSD...VIDLADPKVLPYLPEASRTCM  246

PpPas7p         LCLSYMTNPTAANCGHCFCWSCIIDWCKERQTVLCVGKKCWNSNCYHCIRLFYIPTLNKI  359
                |||| | :|: ::||| ||| |::|| ||||             |  |
HpPer8p         LCLSPMKDPSCGECGHVFCWKCVLDWVKERQE............CPLC.............  282

PpPas7p         LFFFLLSFLSVRAASKEFKSTKEEFAELFNEELADIAGEDPHCPGPFPTGRTLGYFLVVF  419
                           ||  :|        ::|:                |:
HpPer8p         ...........RAKMRE........SQLL.................PLR            295
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FIG. 13. Alignment of the deduced amino acid sequences of *P. pastoris* Pas7p (PpPas7p) and *H. polymorpha* Per8p (HpPer8p). Colons denote similar amino acids, and vertical bars denote identities.

incubation in methanol- or fatty acid-containing media, the peroxisome proliferation response must be intact even in the absence of Pas7p. Thus, we can conclude that Pas7p is not essential for peroxisome proliferation. Also, recruitment of lipids to form peroxisome membranes appears to be intact in the *pas7*Δ mutant.

Methanol-induced wild-type cells occasionally contained peroxisomes in invaginations of the vacuolar membrane that were similar to those observed in the *pas7*Δ mutant. These putative peroxisomal intermediates consisted of flattened membrane sheets that differed from the peroxisome ghosts of the *pas7*Δ mutant in that they were never observed in clusters and often contained bulbous ends near their termini (Fig. 8F). It will be interesting to determine whether these structures are indeed intermediates of the peroxisome biogenesis pathway. If they are, then the fact that sheets of peroxisomal membranes have also been observed in regenerating rat liver (49) and in *S. cerevisiae* during growth on oleic acid (26, 53) suggests that peroxisome assembly in other organisms may occur via a similar intermediate structure.

The zinc-binding domain of Pas7p. Mutational analysis of the C_3HC_4 domain in Pas7p, together with zinc-binding studies on this domain, indicate that zinc binding is essential for the activity of Pas7p. Applying the structural model of the C_3HC_4 domain from Vmw110 protein (2) to the C_3HC_4 domain of Pas7p, cysteines 298, 301, 318, and 321 of Pas7p (the first, second, fifth, and sixth zinc-binding residues) would coordinate one zinc ion while cysteine 313, histidine 315, and cysteines 344 and 347 of Pas7p (the third, fourth, seventh, and eighth zinc-binding residues) would coordinate a second zinc ion. The resulting structure would resemble a coiled ring, with the region between cysteines 321 and 344 forming an α-helical loop. A prediction of this model is that each of the inactivating point mutations (C313S, H315W, and C318S) would disrupt only one of the zinc-binding clusters and that each mutant protein would retain one-half of its zinc-binding activity. Consistent with this model, we observed that the MBP-Pas7p/C313S fusion protein, which would be predicted to lose one zinc-binding site, had approximately one-half the zinc-binding activity of wild-type MBP-Pas7p. Also, MBP-Pas7p/C316S, which retains all conserved zinc-binding residues, had a zinc-binding capacity similar to that of wild-type MBP-Pas7p.

Our observation that the conserved zinc-binding residues are essential for Pas7p is similar to what has been observed for mammalian PAF-1, a distinct peroxisome assembly factor (it is the mammalian ortholog of the *P. pastoris PER6* gene product [8a]). In PAF-1 from CHO cells, substitution of a tyrosine for a conserved zinc-coordinating residue of the PAF-1 C_3HC_4 domain abolishes protein import into peroxisomes (75). However, transfection experiments have revealed that mutant PAF-1 proteins lacking the zinc-binding domain of the protein can suppress the protein import defects of PAF-1-deficient human cells homozygous for a nonsense mutation in the PAF-1 gene (67, 78). The fact that expression of the peroxisomal membrane protein PMP70 can also suppress the same molecular defects in the PAF-1 gene in human cells (26) in similar transfection experiments suggests that heterologous transfection assays may be detecting high-copy suppression in addition to true complementation.

The C_3HC_4 domain of Pas7p is clearly important for the activity of the protein, but we do not yet know its role. Because so many zinc-binding proteins are involved in nucleic acid binding (3) and so many C_3HC_4 domain-containing proteins are involved in nuclear processes (42), it has been proposed that the C_3HC_4 domain is a DNA-binding motif (2, 17, 48, 59). However, our finding that Pas7p is an integral peroxisomal membrane protein with its C-terminal zinc-binding domain inside the peroxisome, combined with the fact that peroxisomes contain no nucleic acids (44), strongly suggests that nucleic acid binding can be safely ruled out as a possible function for the zinc-binding domain of Pas7p. Determining whether this region of Pas7p is involved in protein or lipid binding is clearly important, given the many examples of zinc-binding domains mediating protein-protein or protein-lipid interactions (6, 10, 20, 21, 23, 46, 58, 62). However, it is also possible that the C_3HC_4 domain in Pas7p does not mediate interaction with another molecule but, rather, is required for Pas7p to attain its proper tertiary structure.

Conclusions. We have shown that Pas7p is a zinc-binding integral membrane protein necessary for peroxisome assembly, essential for import of both PTS1 and PTS2 proteins, and required for establishment of the peroxisome lumen. The fact that loss of a single factor could prevent formation of the peroxisome lumen has important implications for peroxisome assembly. If the primary role of Pas7p is in protein translocation, the import of all matrix content must then be tied to protein translocation. Alternatively, if the role of Pas7p is simply to form the lumen, this would imply that protein translocation across the peroxisome membrane is dependent upon a prior step in peroxisome assembly other than formation of the peroxisome membrane.

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C.T. and J.E.K. contributed equally to the work described in this article.

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