

***PEBI (PAS7)* in *Saccharomyces cerevisiae* Encodes a Hydrophilic, Intra-peroxisomal Protein That Is a Member of the WD Repeat Family and Is Essential for the Import of Thiolase into Peroxisomes**

Jing Wei Zhang and Paul B. Lazarow

Department of Cell Biology and Anatomy, Mount Sinai School of Medicine, New York 10029

Abstract. We have previously described mutant *S. cerevisiae* that are defective in peroxisome biogenesis (*peb* mutants) (Zhang, J. W., Y. Han, and P. B. Lazarow. 1993. *J. Cell Biol.* 123:1133–1147.). In some mutants, peroxisomes are undetectable. Other mutants contain normal-looking peroxisomes but fail to package subsets of peroxisomal proteins into the organelle (Zhang, J. W., C. Luckey, and P. B. Lazarow. 1993. *Mol. Biol. Cell.* 4:1351–1359.). In *pebl (pas7)* cells, for example, the peroxisomes contain proteins that are targeted by COOH-terminal tripeptides and contain acyl-CoA oxidase (which is probably targeted by internal oligopeptides), but fail to import thiolase (which is targeted by an NH₂-terminal 16-amino acid sequence). These and other data suggest that there are three branches in the pathway for the import of proteins into peroxisomes, each of which contains a receptor for

one type of peroxisomal topogenic information. Here, we report the cloning and characterization of the *PEBI* gene, that encodes a 42,320-Da hydrophilic protein with no predicted transmembrane segment. The protein contains six WD repeats, a motif which has been found in 27 proteins involved in diverse cellular functions. The *PEBI* gene product was tagged with the hemagglutinin epitope and found to rescue thiolase import in the *pebl* null mutant. The epitope-tagged protein was shown to be inside of peroxisomes by immunofluorescence, digitonin permeabilization, equilibrium density centrifugation, immunoelectron microscopy, and proteinase K protection studies. The *PEBI* gene product does not cleave the thiolase-targeting sequence. It may function to draw thiolase into peroxisomes.

THE peroxisome is an almost ubiquitous organelle in eukaryotic cells. It serves varied functions in different species and cell types. There are two functions, namely H₂O₂-based respiration and fatty acid oxidation, which are shared by peroxisomes from nearly all species. In mammalian cells, peroxisomes also participate in plasmalogen synthesis, cholesterol synthesis, and bile acid synthesis (for reviews see references 31, 35, 65). In human beings, the normal function of peroxisomes is required for the survival of the individual. Human patients born with Zellweger syndrome, which results from defective peroxisome biogenesis, die within a couple of months (35).

It is currently believed that peroxisomes grow by the import of newly synthesized proteins from the cytosol and that new peroxisomes are formed by fission of preexisting organelles (5, 34, 59). Three types of topogenic sequence have been characterized for the import of peroxisomal matrix pro-

teins. A noncleavable, carboxy-terminal SKL tripeptide (and its variants) functions to target proteins including catalase into peroxisomes in many diverse species (18, 28). The rat peroxisomal enzyme thiolase uses an amino-terminal cleavable oligopeptide for its targeting (43, 60). A similar NH₂-terminal peptide is used by thiolase in *Saccharomyces cerevisiae* but is not cleaved (10, 16). Recent evidence indicates that thiolase can enter peroxisomes as a dimer (15). The acyl-CoA oxidase from *Candida tropicalis* has two redundant internal topogenic oligopeptides (26, 57). Internal targeting information is also present in *S. cerevisiae* catalase A (30) and possibly in human alanine glyoxylate aminotransferase (46).

The fact that more than one type of topogenic sequence is used by peroxisomal proteins leads to the speculation that there may be three receptors, one for each type of topogenic feature. Studies on human genetic diseases and peroxisome biogenesis mutants in yeast have supplied evidence consistent with this hypothesis (45). Fibroblasts from one complementation group of Zellweger patients are unable to import proteins with SKL-type sequences into peroxisomes but thiolase is packaged inside the organelle (41). On the other hand, peroxisomes in fibroblasts from patients with the rhi-

Address all correspondence to Jing Wei Zhang, Department of Cell Biology and Anatomy, Box 1007, Mount Sinai School of Medicine, One Gustave Levy Place, New York, NY 10029-6574. Tel.: (212) 241-1749. Fax: (212) 860-1174.

zomelic type of chondrodysplasia punctata have normal packaging of catalase, acyl-CoA oxidase and multifunctional protein but the packaging of thiolase is defective (21). Zellweger patients from many complementation groups assemble peroxisomal membranes but do not package any of the peroxisomal matrix proteins, indicating that some gene products are required for the import of all peroxisomal matrix proteins, regardless of the type of topogenic information (35, 41, 73).

We have isolated and characterized peroxisome biogenesis (*peb*) mutants from *S. cerevisiae*, among which two have normal-looking peroxisomes but are selectively defective in the import of one subset or another of peroxisomal proteins (79, 80). *Pebl-1* does not package thiolase, but the import of catalase and acyl-CoA oxidase is normal; *peb5-1* does not package catalase, but the thiolase and acyl-CoA oxidase are in peroxisomes. Based on these results and other data reported below, we proposed that there are three pathways, or three branches of a pathway, involved in the import of peroxisomal proteins (80). Other groups have reported the isolation of similar yeast peroxisome assembly (*pas*) mutants (32, 40, 67). *Pas7*, from *S. cerevisiae*, is selectively defective in thiolase packaging and we learned after the initial submission of this manuscript that *pas7* (37) is defective in the same gene as *pebl*. *S. cerevisiae pas10* and *P. pastoris pas8* are selectively defective in packaging luciferase and other proteins targeted by -SKL. The defective gene product in the latter mutant is capable of binding peptides that end in SKL, suggesting that it may be an -SKL receptor.

In the present study, we have investigated the gene that is defective in the *pebl-1* mutant. The defect causes thiolase, that is normally localized within peroxisomes, to be mislocalized to the cytosol. This is observed experimentally as a change from punctate fluorescence with anti-thiolase to diffuse, bright fluorescence throughout the cells (79). It is also observed by the selective permeabilization of spheroplasts with progressively increasing concentrations of digitonin: whereas thiolase emerges from wild-type yeast together with the other peroxisomal enzymes at digitonin concentrations of 300–600 $\mu\text{g/ml}$, thiolase is released from *pebl-1* with the cytosolic enzymes at much lower digitonin concentrations (80). The *pebl-1* phenotype resembles wild type in that normal-looking peroxisomes are present, which contain catalase and acyl-CoA oxidase and probably most other peroxisomal enzymes, as assessed by electron microscopy, immunogold labeling, cytochemistry, cell fractionation, and digitonin permeabilization.

Thiolase carries out an essential catalytic function in the β -oxidation of fatty acids (the thiolytic cleavage of acetyl-CoA from β -keto-acyl-CoA). β -oxidation occurs solely in peroxisomes in *S. cerevisiae* (70). The mislocalization of thiolase to the cytosol in *pebl-1* prevents this mutant from β -oxidizing fatty acids. This is seen experimentally as an inability to grow on plates containing oleic acid as the carbon source.

Because *pebl-1* is selectively defective in importing thiolase into peroxisomes, we suspected that the *PEBI* gene might encode a thiolase receptor, perhaps located on the cytosolic surface of peroxisomes, or perhaps soluble in the cytosol. Here we report the cloning and characterization of the *PEBI* gene. We found that *PEBI* encodes a 42,320-Da hydrophilic protein that is a member of the WD repeat family. Unexpectedly,

this protein is located within the peroxisome. The possible role of the protein encoded by the *PEBI* gene (*Peblp*) in peroxisome biogenesis is discussed.

Materials and Methods

Yeast Strains, Plasmids, and Culture Conditions

Yeast strains used in this study are listed in Table I. These strains contain active catalase A (the peroxisomal isozyme) but defective catalase T (the cytosolic isozyme) (*ctl-1*). The plasmids are listed in Table II. To test the ability of cells to utilize oleic acid, plates containing 0.05% yeast extract, 0.67% yeast nitrogen base without amino acids, 0.1% oleic acid, and 0.25% Tween 40 (YNO) were used (11). For most experiments, yeast were precultured twice in rich glucose medium (YPD) and then grown for 18 h in a rich medium containing glycerol and oleic acid (YPGO) as described (80). Glycerol supports the carbon requirement of the yeast, regardless of whether the peroxisomes are functional, and oleic acid causes peroxisome induction. Plasmid-containing strains were precultured twice in minimal medium plus glucose and arginine to prevent plasmid loss. YPD, minimal media, and synthetic complete medium were prepared as described (54).

Isolation of *PEBI* Gene

The mutant *pebl-1* was transformed with a Ycp50-based genomic library constructed by Rose et al. (47) that contains a *URA3* marker. About 1×10^9 cells were spread on synthetic complete plates lacking uracil. The Ura+ transformants were screened for the ability to grow on YNO: ~8–10 transformants were pooled and resuspended in 100 μl of sterile water in a well of 96-well plates. These were then transferred to YNO plates using a multi-prong inoculator (frogging technique). Plasmid DNA from a transformant that grew on YNO was isolated according to Hoffmann and Winston (23) and transformed into *E. coli* by electroporation (7). Plasmid DNA was prepared from three independent bacterial transformants; restriction mapping indicated that each contained the same 10-kb insert.

DNA Sequencing

Plasmid pR2 (Table II), which contains a 3-kb fragment that complements the *pebl* mutation, was subjected to progressive shortening with exonuclease III (22). The nested subclones were sequenced by the dideoxy (Sanger) sequencing method (51) using T3 primer. The few gaps in this strand and the opposite strand were sequenced using synthesized oligonucleotides as primers.

1. *Abbreviations used in this paper:* HA, hemagglutinin; ORF, open reading frame; *pas*, peroxisome assembly; *peb*, peroxisome biogenesis; *Peblp*, protein encoded by the *PEBI* gene; UTR, untranslated region; YPD, rich glucose medium; YPGO, rich medium containing glycerol and oleic acid; YNO, plates containing yeast extract, yeast nitrogen base without amino acids, oleic acid, and Tween 40.

Table I. Yeast Strains Used in This Study

Name	Genotype	Sources
JW68-3A (wild type)	<i>MATα, ura3-1, trp1-1, arg4, ctl1-1</i>	Reference 79
<i>pebl-1</i>	<i>MATα, pebl-1, ura3-1, trp1-1, ctl1-1</i>	Reference 79 (m6-D1)
<i>pebl-1-R1</i>	<i>MATα, pebl-1, ura3-1, trp1-1, ctl1-1 [pR1]</i>	This study
JW75*	<i>MATα, ura3-1, trp1-1, arg4, ctl1-1, pebl::TRP1</i>	This study
JW86*	<i>MATα, ura3-1, trp1-1, arg4, ctl1-1, pebl::TRP1 [YipPEB1-HA₃]</i>	This study
JW88*	<i>MATα, ura3-1, trp1-1, arg4, ctl1-1, pebl::TRP1 [pBXNPEB1-HA₃]</i>	This study

* Isogenic with JW68-3A.

Table II. Plasmids Used in This Study

Name	Characteristics	Source
pR1	10-kb fragment complementing the <i>peb1-1</i> mutant. Came from the genomic library (reference 47). URA3. Ycp50 based.	This study
pR2	3-kb fragment complementing <i>peb1-1</i> . Cloned in pRS314 (see below)	This study
pRSPEB1	<i>PEB1</i> ORF including 53-bp upstream untranslated sequence cloned in pRS314. T3 promoter.	This study
pRSPEB1-HA ₃	<i>PEB1</i> ORF including 53-bp upstream untranslated sequence tagged with three copies of the HA epitope cloned in pRS306. T3 promoter.	This study
YipPEB1-HA ₃	Triple-tagged <i>PEB1</i> including 1110-bp upstream untranslated sequence cloned in pRS306.	This study
pBXNPEB1-HA ₃	Triple-tagged <i>PEB1</i> ORF including 53-bp upstream untranslated sequence cloned in pAB23BXN. GAPDH promoter. 2 μ .	This study
pJW17	<i>peb1::TRP1</i> . The Bgl2 fragment of the <i>PEB1</i> gene was replaced with the <i>TRP1</i> gene. Based on pR2. See Fig. 2 D.	This study
pRB58	A 12-kb genomic fragment containing <i>POT1</i> (thiolase gene) and <i>SUC2</i> , cloned in Yep24.	Reference 6
pRSThiolase	The thiolase ORF (subcloned from pRB58) containing 87-bp upstream untranslated sequence, cloned in pRS316. T3 promoter.	This study
pRSThiolase Δ 1-16	The thiolase ORF with deletion of sequence encoding the first 16 amino acid residues, cloned in pRS316. T3 promoter.	This study
pRS314	<i>TRP1</i> . CEN4.	Reference 55
pRS306	<i>URA3</i> . Yip.	Reference 55
pRS316	<i>URA3</i> , CEN4.	Reference 55
pAB23BXN	<i>URA3</i> . GAPDH promoter. 2 μ	Reference 52

In Vitro Transcription and Translation

The reagents and protocols for the in vitro expression of proteins were from Promega Corp. (Madison, WI). Plasmids pRSPEB1, pRSPEB1-HA₃, PRSThiolase, and PRSThiolase Δ 1-16 were transcribed with T3 RNA polymerase after linearization. RNA was extracted with phenol/chloroform, ethanol-precipitated, and translated in vitro with reticulocyte lysate and [³⁵S]trans-label (New England Nuclear, Boston, MA). SDS-PAGE and fluorography were as described (20). Molecular mass standards were a mixture of "rainbow" markers and [¹⁴C]methylated markers (CFA7561; Amersham Corp., Arlington Heights, IL); in the instances where the colored protein migrates slower than the radioactive standard, the position of the latter was used.

Epitope Tagging

The nine-amino acid epitope of influenza virus hemagglutinin (HA) (YPY-DVPDYA), which can be recognized by the monoclonal antibody 12CA5 (75), was used for tagging. It is referred to hereafter as the HA epitope. Three tandem copies of the HA epitope, separated by one or two spacer amino acids, were added to the COOH terminus of the *PEB1* gene product by two consecutive PCR reactions (29).

In the first PCR reaction, one HA epitope was added to the extreme COOH terminus of the wild-type *PEB1* gene with the following primer: 5' GTG TCA AGC GTA GTC TGG GAC GTC GTA TGG GTA ACC TAA GCC GTT CCA TAC AAA TA 3'; underlined bases are complementary to the plus strand of the *PEB1* gene with the stop codon in bold type. The second primer consisted of nucleotides 79–86 of *PEB1*. The PCR product was cloned and sequenced.

In a second PCR reaction, two additional copies of the HA epitope, separated by one or two spacer amino acids (64) were added to the end of the *PEB1* gene. The intended oligonucleotide sequence was 5'TCA TTA GGC G(T)A ATC AGG AAC GTC ATA CGG GTA GGA TCC TGC ATA GTC CGG TAC ATC ATA GGG ATA GCC AGC GTA GTC TGG GAC GTC GTA 3', with the sequence encoding the HA epitope being underlined. The third base in many codons was changed in the second and third epitopes to avoid inappropriate self-annealing. The same internal primer that was used in the first PCR reaction was also used in this reaction.

The PCR product was cloned and digested with Bgl2. The downstream portion of it was used to replace the corresponding part of the wild-type copy of the *PEB1* gene (see Fig. 2 C), generating the triple-tagged version. Sequence analysis revealed that the *PEB1* gene fragment synthesized by PCR was 100% correct, but that the 11th nucleotide, T, in the second primer (in parentheses above), was omitted during commercial synthesis of the oli-

gonucleotide. This error produced a frame shift that resulted in an incomplete third epitope, consisting of seven instead of nine amino acids, followed by two erroneous and four extra amino acids and a new stop codon (from the polylinker). The final sequence of the COOH terminus of the triple-tagged Pebp was GLGYDYDVPDYAGYPYDVPDYAGSYDYDVPDPSN-ESI, with the epitope sequence being underlined.

Cell Fractionation

Yeast cells grown in YPGO were converted to spheroplasts as described previously (80). Homogenization of spheroplasts, differential centrifugation, and equilibrium density centrifugation of a crude organelle fraction in a linear Nycodenz gradient were carried out according to Lazarow et al. (36).

Proteinase K Treatment of Organelles

A crude organelle fraction was subjected to gentle proteolysis as described by Hohfeld et al. (24) with minor modifications. The 25,000-g pellet fraction from YPGO-grown JW88 was resuspended in 1.2 M sorbitol, 5 mM MES, pH 6.0, and divided into aliquots. The organelles (0.5 mg/ml final concentration) were incubated in a 50- μ l vol with proteinase K coupled to agarose beads (P 9290; Sigma Chem. Co., St. Louis, MO) (25 mg/ml) in the absence and presence of 0.1% Triton X-100 at room temperature with gentle rotation. The digestion was stopped at different time points by the addition of 1 μ l of 100 mM PMSF in ethanol.

Immunoelectron Microscopy

The electron microscopic immunolocalization with gold particles was according to Wright and Rine (76), using 12CA5 at a dilution of 1:250, and protein A conjugated to 20 nm gold at 1:50. Double-immunolabeling was according to Varndell and Polak (69), with 12CA5 at a dilution of 1:100, rabbit anti-thiolase at a dilution of 1:100 and 10-324 at 1:200. 10-324 is a rabbit antiserum raised against total peroxisomes of *Candida tropicalis* (56), that cross-reacts with *S. cerevisiae* peroxisomal proteins (61). Secondary antibodies (EY Laboratories, San Mateo, CA) were goat anti-mouse IgG (Fc fragment specific) conjugated to 20 nm gold, and goat anti-rabbit IgG (Fc fragment specific) conjugated to 5 nm gold, used at dilutions of 1:50 and 1:100, respectively. Control experiments demonstrated that the secondary antibodies did not cross-react inappropriately with the primary antibodies.

Other Methods

The progressive permeabilization of yeast membranes with digitonin (80),

immunofluorescence (79), the catalase assay (2), and the fumarase assay (3) were according to described methods. Immunoblotting was carried out with the enhanced chemiluminescence method (Amersham Corp.) as described previously (80) (Figs. 8, 10, 11) or with [¹²⁵I]Protein A (5 × 10⁵ cpm/ml, Fig. 13). Immunoblot data were quantitated with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA) (80). The DNA recombination techniques were generally from Sambrook et al. (49). The yeast genetics were according to Sherman (54).

Materials

Nycodenz was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). Digitonin (lot no. 113H0889) was from Sigma (D1407). The monoclonal antibody 12CA5 that recognizes the HA epitope was kindly provided by Dr. Michael Shia (Boston University School of Medicine). Rabbit anti-thiolase and rabbit anti-phosphoglycerate kinase were generous gifts from Dr. Wolf Kunau (University of Bochum, Bochum, Germany) and Dr. Jeremy Thorner (University of California at Berkeley), respectively. The oligonucleotides were synthesized by the Mount Sinai DNA Core Facility. Other reagents were from Promega (Madison, WI), Sigma or New England Biolabs (Beverly, MA).

Results

Cloning of *PEB1* by Functional Complementation

The *peb1-1* mutant, which is unable to grow on oleic acid due to the mislocalization of thiolase from peroxisome to

cytosol, was transformed with a yeast genomic library (47). About 3,000 transformants were obtained, of which three were able to grow on oleic acid (Fig. 1 *A*). After subcloning, samples of the three transformants were grown in non-selective, glucose-containing (YPD) medium overnight to allow plasmid loss. In the case of one transformant, *peb1-1-R1*, those cells that lost the plasmid also lost the ability to grow on oleate. The other two transformants continued to grow on oleate after plasmid loss and were discarded.

Immunofluorescence analysis of *peb1-1-R1* (containing plasmid) with anti-thiolase showed punctate staining (Fig. 1 *B*), indicating that this transformant had also regained the ability to package thiolase within peroxisomes. Plasmid pR1 was retrieved from this transformant and found to contain a 10-kb insert. Plasmid pR1 was transformed back into *peb1-1*, where it restored thiolase packaging and peroxisomal function to the mutant, as seen by growth on YNO medium and immunofluorescence with anti-thiolase (data similar to Fig. 1). The restriction map of the insert is shown in Fig. 2 *A*.

Defined restriction fragments of the insert were subcloned into pRS314. These subclones were transformed back into *peb1-1* and tested for their ability to complement the *peb1* mutation. The smallest fragment with the ability to restore the growth of the mutant *peb1-1* on a YNO plate was a 3-kb

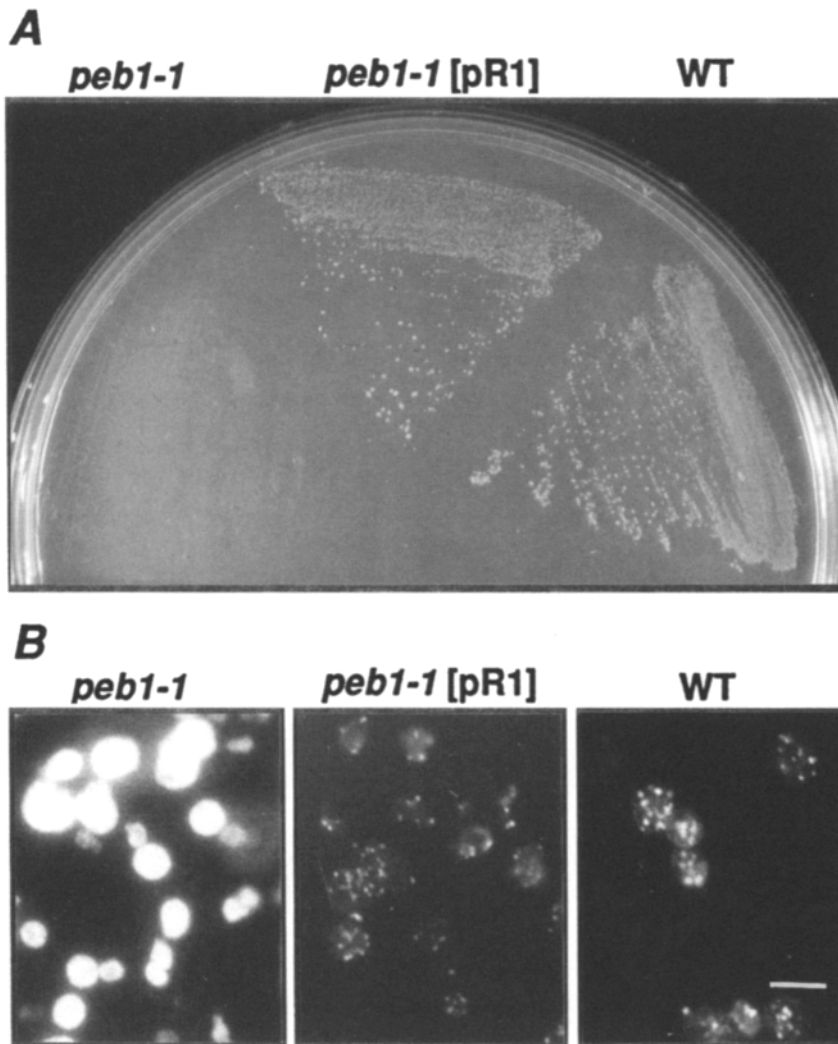


Figure 1. Functional complementation of the *peb1-1* mutant. (*A*) The growth on oleic acid (YNO plate) of *peb1-1*, *peb1-1* that had regained the ability to grow on YNO after transformation with a plasmid from a genomic library (*peb1-1 [pR1]*), and wild type (JW68-3A). (*B*) Immunofluorescence with anti-thiolase of cells grown in YPGO medium to induce peroxisomes (see Methods). Bar, 5 μ m.

SacI-SmaI fragment. This fragment also had the ability to restore the punctate type of fluorescence with anti-thiolase to the mutant (Fig. 2 A).

Sequence Analysis

The 3-kb fragment was sequenced (Fig. 2 B). It contained an open reading frame (ORF) of 1275 bp, corresponding to a putative protein of 375 amino acids with a molecular mass of 42,320 Da (Fig. 3). No intron was identified. The nucleotide at the -3 position relative to the first ATG is A, which is strongly conserved in yeast genes (63). The C at the +4 position is less common. In vitro transcription and translation of this plasmid produced a labeled protein of ~43 kD

(Fig. 4) consistent with the calculated molecular mass. These results confirmed that the first ATG in the ORF of Fig. 3 is the translation start site.

The nucleotide sequence from -238 to -214 resembles an oleate-response element or "peroxisome box" (8, 12, 72) that may regulate the expression of this gene. A polyadenylation site (AATAAA) was seen +270 to 276 bp down stream of this ORF (Fig. 3). The nucleotide sequence of the *PEB1* gene was compared with the EMBL/GenBank nucleotide sequence data base (updated July 6, 1994) with the FASTA search of the GCG sequence analysis program (version 7). No significant sequence similarity to any known gene was found. Very recently we learned that *PEB1* = *PAS7* (37).

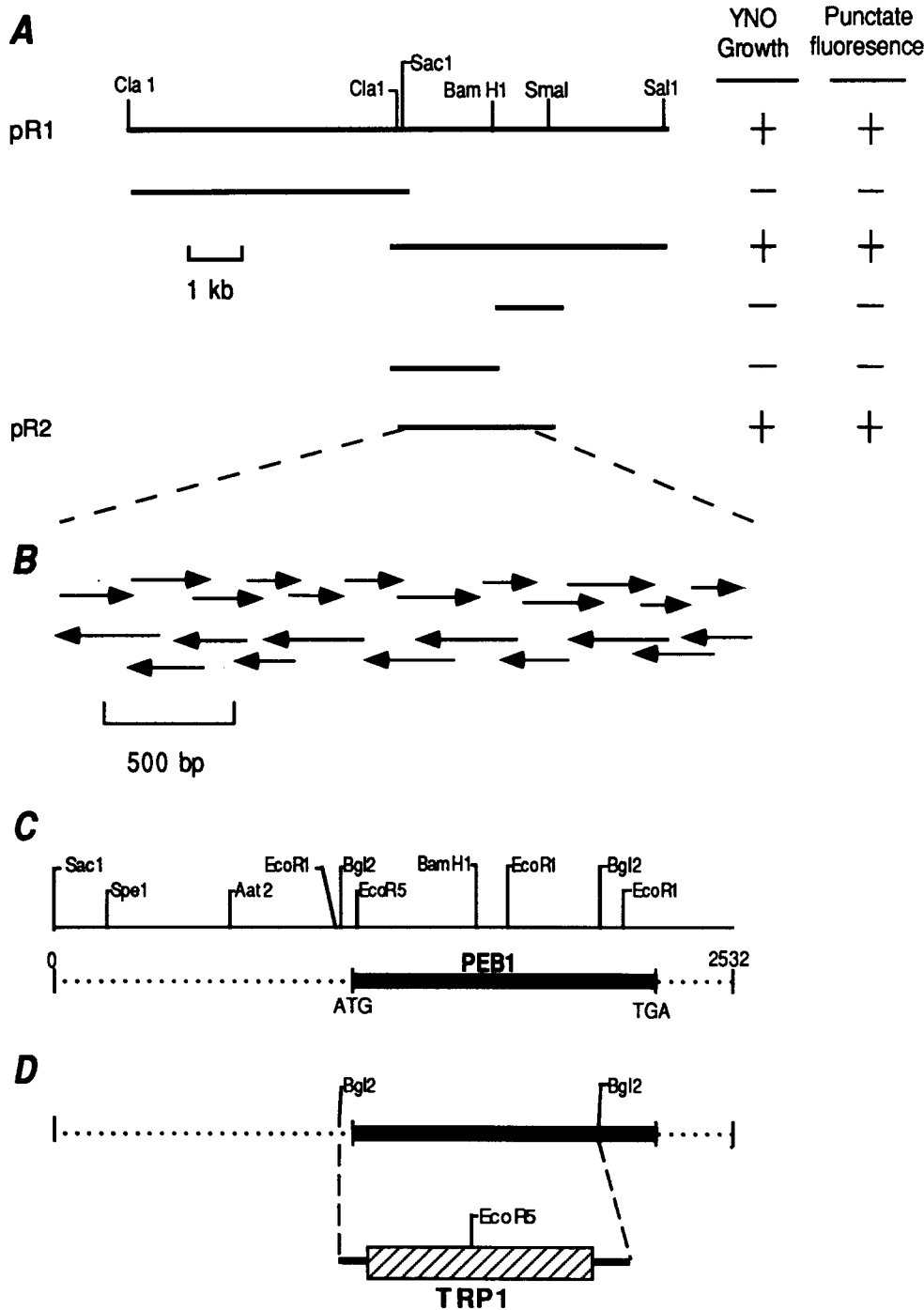


Figure 2. Localization of the *PEB1* gene within the plasmid pR1, sequence strategy, and gene knockout strategy. (A) The ability (+, -) to complement the *pebl-1* mutant with fragments of the 10-kb genomic DNA insert in plasmid pR1. Complementation was tested by growth on oleate plates (*YNO Growth*) and immunofluorescence with anti-thiolase (*Punctate IF*). These two tests gave consistent results. (B) The direction and extent of sequence determinations of pR2 are indicated by arrows. (C) A detailed restriction endonuclease map of the sequenced region and the identified ORF. (D) Strategy for disruption of the *PEB1* gene. The *Bgl2* fragment of the *PEB1* gene, which contains the majority of the *PEB1* ORF, was replaced by the *TRP1* gene. The scale of C and D is the same as B.

-1110

-1080 TCTGGTGTGGATACAACTAAAGTCCGCC TGGTCCCATCCCTTTCATCTTTCCTCAAT TCCAGCTCCAAATCGAATCTCCGCTT -1081
 -990 CTGATCTTCGCAAGCCATCAGCAGGAA CCGTCCAACTCAAAATACACTAGTAGA CGCTACAGCCCTCCACACACAGCTAG -991
 -900 AGCGTGTGGATCACTATATCAAGCTTGC TCAGAGGGAACTCTGATACCTCTGGAAC AACAGCCCGGAGCAGCAAACTCTCACAA -811
 -810 CCAAGAGGTATGACTATCGCTCTCTGA TACACCCAGGAGTCTCTTTTGGAAAA TATTTACAGATTAAAGGCCATTTACGAA -811
 -720 TGGCTCAGAGCAACATACGCAATATTA CCAATAGCAATGATCAGCAGGCTGGAG TCTGTGTGAGACACAGAAATACCTGTT -811
 -630 TTGCTCCGCTGTGCGGATATAGAAATCA AATGCTGATCTTACTGCTGGATGAC CACACAAATCTCCGCTCTCTCTAC -811
 -540 AGACAAATCTCTCAAGGCCAAGCTTGC AACGCTTCGGACCCACCACTCATACGA CGTCCCTAATGATCAATGAGCAGCTC -811
 -450 ATCAAAATGAAATACCGCTCTGAGTA TGTATATGATGATATCTCTTATTTCTTAC TCTACTTAAAGTCTATATCAATCTACTC -811
 -360 TTTTACCTTTTTTATCTGCACTTTATA TATATATGATGATATCTCTTATTTCTTAC TCTACTTAAAGTCTATATCAATCTACTC -811
 -270 TCAGAAAATTTGATCTCCAGCAAGTAA TATGATGATGATGATATCTCTTATTTCTTAC TCTACTTAAAGTCTATATCAATCTACTC -811
 -180 CAGTAAAGGTGAAAGCACTTTTAAAGACT GTGAGACAAAGCTGTGTTTCTTACGATA AATCTTTTACCGGAGTGGAAATGCTTTT -91
 -90 CTTTCTGATTTGGAATGGAGCTGAAAA CTGACAGATCTCATATTTTAAAGGACTA TCATCTAATCTTTGCTATATTTATACAC -1

1 ATGCTCAGATCATATGCAAGGTTTACT GGGTACGGTCTCCGATTTCCCTTTTTC GATAACGGCTCGGGGTACTGCTGGTTCA 90
 1 M L R Y H H M Q C F S G Y C V Q V Y S P F F D N R L L A V A A G S 30
 91 AACCTTGGCTGGTGGGAAAGTATTTTTC TTACTCTGAGATTTGATCTGAGTGA ATATGGAGTAACTCCCTTTTAAACAG 180
 31 N P F G L V F Q N K L F I L E I D R S C R I V E V N S F L T Q 60
 181 GATTTGTATTTGATCTGATGGAACGAA NGTATGAAACCAAGTGTGGTGCACAG GCGCATGGTACATTTACGCTTTTGTATCA 270
 61 D C L F D L L A N N E S E E N O V L V A Q G D G T L R L F L Q 90
 271 ACCTTTAAGAGTTTCTTATGCTATATTT AAAGAGCATGAGGAGAGTATTCAGTGT AATGGAACTTAGTCAACAGCAAGATTTTC 360
 31 T F R E P P I A I F K E B E R E V F S C N H N L V N R R Q 120
 361 TTAMAGTTCATGGAGTGAATCTAATAA ATATGTCCTCCCTCTAGAAACAAATTTA ATGACCTTACTCCAGCACTTTAGAGATT 450
 121 L S S S M D G S I K I N S P L R K Q S L M T L T P R P L E I 150
 451 ACCAAAATGGTGGATCATTAAAGCCATT ATATGAAACCAAGGCTTTTACAGTATT TCAAAAACAGGACTGTGTATACCAAGCA 540
 151 T R M V D P L N A I L R K R S F T G I S K N R N C V T Y Q A 180
 541 CAGTTCCTGCGCAGCAAAATCTCGTA TTATCTGTTTCCAGGAACTCTTATGCAAGC TTATTTGACATTAAGTACTCTCCGGAAA 630
 181 Q P S P R D Q N L V L S C S G N S Y A S L F D I R L P S G K 210
 631 AATCAGATAATTTTATGCTATCAGGA CTAGAGCACTTGCATGATTTCAACAAA TACAGACCTTATGATTTGCCACAGGAGT 720
 211 N Q N M F L V S S G L E A L T C D F N K Y R P Y V V A T G G 240
 721 CTGATATGCGATTGAACTTGGAGCAAT AGGATGCTAAATAAATGATAGCGACT ATCAAGAGACTGTCCCGGCCAACTCAC 810
 241 V D N A I R I M D I R M L N K N E S A T I K R T V P G Q L H 270
 811 AATTCATCATCATGAAATCCCTAAT GCGCAGGCTGGCAGCAAGAAATTC TGTCCCTCATCATCTCCAATTTTAAAT 900
 271 N S S C I N E I P N A H G L A I R K V T M S P H S H I L M 300
 901 TCAGCTTCATGATGATGCTGCGAATA TGCAGATCTCAGCAGCAAGTGTGCAAA CAACATATAAATCACTACTACGGATGCT 990
 301 S A S Y D M T C R I N R D L S N D G A K E T Y X T N S T D A 330
 991 ACTAAGGTTCCATTTTCAATTTTCAACAG CATTGAGAAATCGTATTTGGAGCTGACTG AGCTTGTGGGAAAGCAGGATATGCTGGCT 1080
 331 T R G S I F N F T Q R S E P F V F G A D W S L W G X P G Y V A 360
 1081 TCACCTGATGGATGCAAAATTTATTTGTA TGCAGCTCTCAGCAGCAATATCTTTT TACACAAATTTTTCGATATTTAAACTT 1170
 361 S T A M D G N L F V M N G L G * 375
 1171 TAAAATTTTTCATATCTGTCAGCAAT TGGCAGATCTTACGTAATTTATTAAT TCTCAACAGAAATTTATGGTAAACAGCT 1260
 1261 TCTGACTGTGCAAAAGCAATCTCAAAAAC ATACCTACCGCAAAAACCTGTAATTACTCA AATTAATCAAGTTTTTATTAACGACATTAAGC 1350
 1351 GAGTGTGAAAGAGGATATTTAGAGTCT GATTACCGTTTATTTTAAACGACATCTT ATATATTGTAAA 1422

Figure 3. Characterization of the *PEBI* gene. Nucleotide and predicted amino acid sequence. The potential polyadenylation site is underlined. The potential peroxisome box or oleate response element is indicated with dashed lines. The stop codon is indicated with an asterisk. These sequence data are available from EMBL/GenBank/DBJ under accession number X83704.

Deletion of the Chromosomal Copy of *PEBI*: Genetic and Phenotypic Analyses

The majority of the ORF was replaced with the yeast *TRP1* gene (Fig. 2 D) and the construct was used to disrupt the genomic copy of the ORF in wild-type strain JW68-3A by one-step homologous recombination (48). The successful disruption of this gene was confirmed by PCR amplification

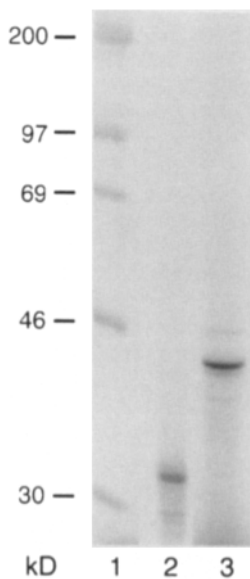


Figure 4. In vitro expression of the *PEBI* gene. pRSPEBI was transcribed with T3 polymerase, translated with reticulocyte lysate, and detected by fluorography (lane 3). Pebp is indicated with an arrow. Molecular mass markers are in lane 1. As a control, the translation products of an unrelated mRNA are shown in lane 2.

of the affected genomic region and restriction nuclease analysis of the product demonstrating the loss of the BamHI site and the appearance of an EcoR5 site (data not shown).

Like the original *pebl-1* mutant, the knockout strain (JW75) could not grow on a YNO plate (Fig. 5 A, center) and thiolase was mislocalized to the cytosol (Fig 5 B, center).

The knockout strain was subjected to digitonin permeabilization. The release patterns of the control enzymes, phosphoglycerate kinase (cytosolic marker), catalase (peroxisomal marker), and fumarase (mitochondrial marker) were almost identical in the *PEBI* knockout (Fig. 6 B) and in wild-type cells (Fig. 6 A). However, thiolase in the knockout strain emerged together with the cytosolic marker, phosphoglycerate kinase, instead of with catalase as in the wild-type cells. These results confirm that the knockout strain, like *pebl-1*, cannot package thiolase into its peroxisomes, although catalase packaging is normal. Luciferase, which is targeted to peroxisomes by a COOH-terminal SKL (18), and which is imported into wild-type *S. cerevisiae* peroxisomes (58) was expressed in the *PEBI* knockout under the control of the *POX1* promoter (72) and found in peroxisomes by immunofluorescence (data not shown).

The *PEBI* knockout strain was crossed with the original *pebl-1* mutant, and the diploid cells thus formed likewise failed to package thiolase into peroxisomes. The diploid was sporulated and four tetrads were dissected and analyzed. All four tetrads showed a 0:4 segregation pattern of thiolase packaging as tested by immunofluorescence with anti-thiolase. These data demonstrate that the cloned ORF is the bona fide *PEBI* gene, and not a suppressor.

Structural Features of the Protein

The deduced amino acid sequence of Pebp was compared with the SwissPro protein data base by the FASTA program of the GCG program. The COOH-terminal two thirds of the protein was similar to several yeast proteins, including *STE4*, *MSII*, and *PWPI*. The regions of similarity with these proteins were confined to WD motifs (also called β -transducin repeats) that all of these genes contain (42, 68). The WD repeat was first identified in the β subunit of heterotrimeric G protein, which consisted of seven such repeats in tandem (13). Proteins with more than two of these repeats have been classified into a family: the members have diverse functions (see Discussion) (42, 68). The consensus sequence of the WD motif is shown in Fig. 7. Pebp contains six WD repeats, which occupy most of the protein except for the first 55 residues (Fig. 7); the extent of mismatch falls well within the range observed for other members of this family.

Hydropathy analysis according to Kyte and Doolittle (33) with a window size of 15 to 19 failed to identify any predicted transmembrane domain. Pebp contains 38% polar residues plus 21% charged amino acids and has an estimated pI of 9.0.

Expression and Function of Epitope-tagged Pebp

Three copies of the nine-amino acid epitope, YPYDVPDYA, of influenza virus hemagglutinin (HA epitope) were added to the extreme COOH terminus of the *PEBI* ORF by means of PCR amplification. Due to a dropped base in a 90-bp oligonucleotide, the third epitope was imperfect (see Materials

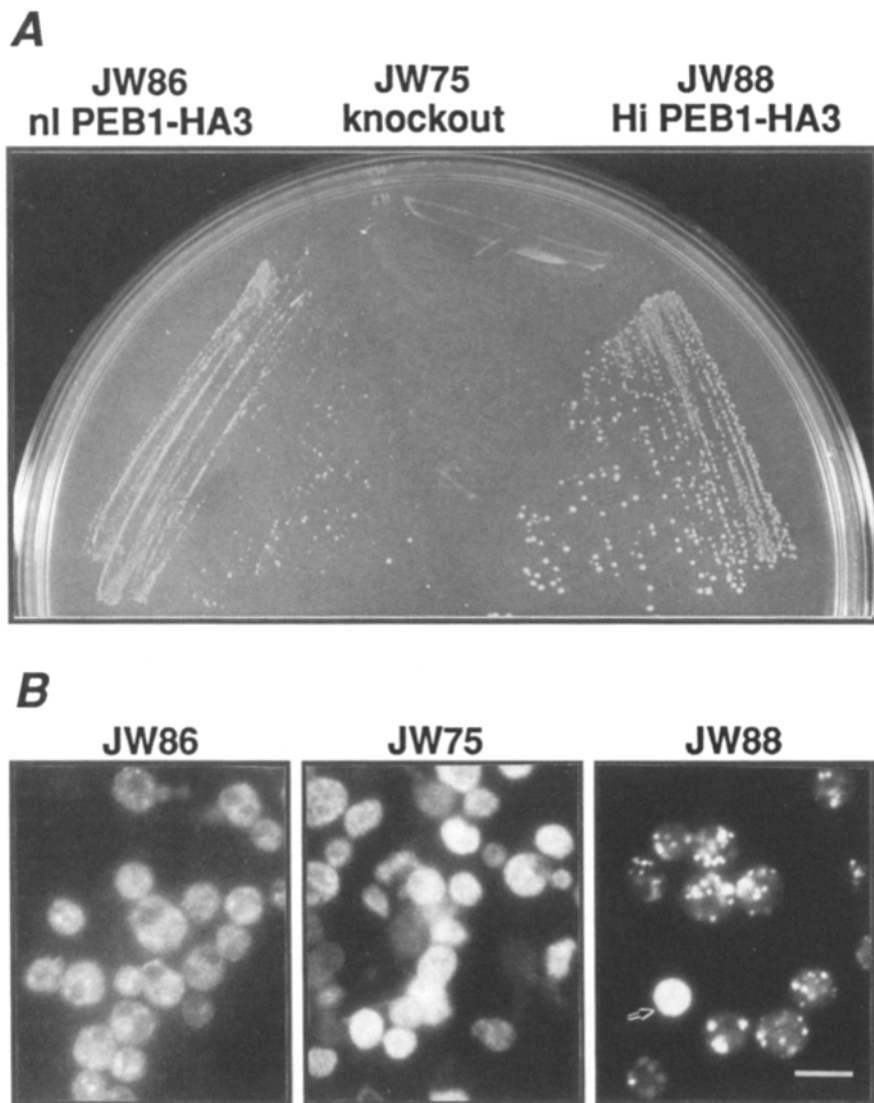


Figure 5. Functional expression of Peblp-HA₃ in the knockout strain. JW75, the *pebl* null mutant. JW86, the null mutant transformed with YipPEB1-HA₃, a single copy integrating plasmid containing *PEBI* with its normal control elements. JW88, the null mutant transformed with pBXN-PEB1-HA₃, a multi-copy plasmid containing *PEBI* under the control of the strong, constitutive GAPDH promoter. (A) Growth on oleic acid. (B) Thiolase immunofluorescence of cells grown in YPGO. Arrow indicates a cell that probably lost the plasmid during growth in the nonselective YPGO medium. Bar, 5 μ m.

and Methods). The triple-tagged *PEBI* ORF (termed PEB1-HA₃) was expressed in vitro (Fig. 8 A) and a band of \sim 48 kD was detected with the epitope-specific monoclonal antibody 12CA5. This is slightly bigger than the predicted mass of the tagged Peblp (46.2 kD); the cause for this difference is not clear.

The triple-tagged *PEBI* ORF with its own promoter (1110 bp of 5' untranslated region [UTR]) was cloned into a single-copy integrating Yip plasmid (YipPEB1-HA₃, Table II) that was transformed into the *pebl* null mutant after linearization of the plasmid in the *URA3* gene. The transformant was called JW86. Yeast transcription control elements are usually located within 500–600 bp of the 5' UTR (19) and this is also true for genes encoding peroxisomal proteins (8). Since JW86 contains one copy of the triple-tagged *PEBI* gene with 1,110 bp of 5' UTR, its expression is assumed to be at the wild-type level. Strain JW86 was grown in liquid medium containing glycerol plus oleic acid to induce peroxisomes (80). A faint band with a mass of \sim 48 kD, the same as the in vitro translation product, was detected with monoclonal

antibody 12CA5 in JW86 but not in the untransformed knockout (JW75) (Fig. 8 B).

The triple-tagged *PEBI* ORF (with 54-bp 5' UTR) was also cloned into a multi-copy episomal plasmid (PAB23BXN) behind the strong, constitutively expressed GAPDH promoter (4, 52); this was transformed into the *pebl* null mutant, generating strain JW88. Expression of the triple-tagged *PEBI* gene in this strain is expected to be high, and indeed an intense band of 48 kD was detected. Two faint bands, one above the major band and one below it, also were seen in JW88; their significance is unknown.

The presence of the tagged protein in strains 86 and 88 did not affect their growth in either YPD or YPGO medium (data not illustrated). Peblp-HA₃ restored the ability of the knockout strain to grow on oleic acid (Fig. 5 A). At high expression levels (JW88) the growth rate was the same as the wild type. However, at a normal expression level, the colony size was smaller, suggesting that the tags may have interfered to some extent with the functioning of Peblp. This inference was supported by an immunofluorescence analysis of the in-

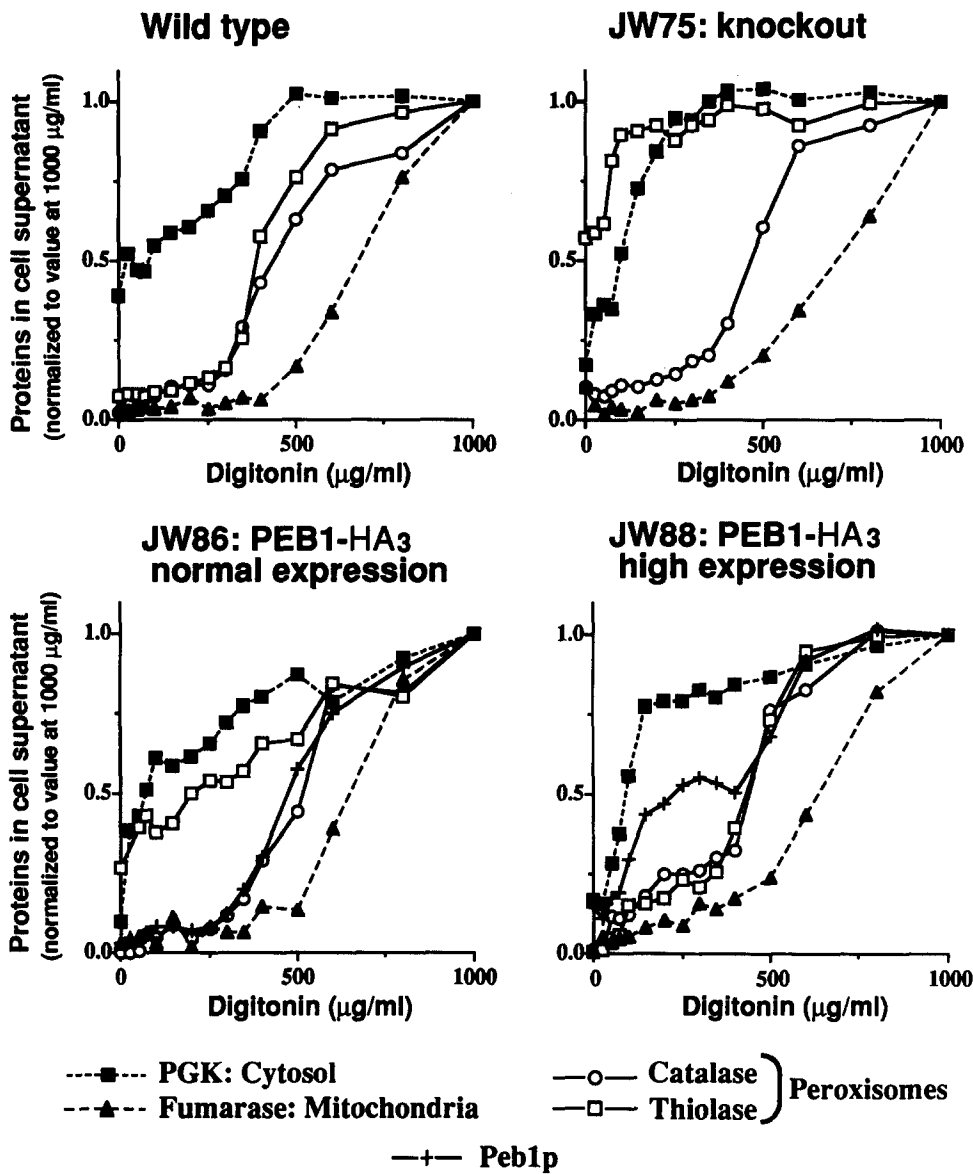


Figure 6. Release of intracellular proteins by digitonin titration. Aliquots of spheroplasts were incubated with different concentrations of digitonin for 20 min at 4°C and then centrifuged to pellet the cells. The supernatants were analyzed for the release of proteins. The activity of the peroxisomal enzyme, catalase, and the mitochondrial enzyme, fumarase, were assayed enzymatically. The cytosolic isozyme of catalase (catalase T) is defective in these strains. The cytosolic marker, phosphoglycerate kinase (*PGK*), as well as thiolase and triple-tagged *Peb1p* were analyzed by immunoblotting. The amount of each enzyme released was normalized to the amount found in the supernatant at 1,000 µg/ml of digitonin.

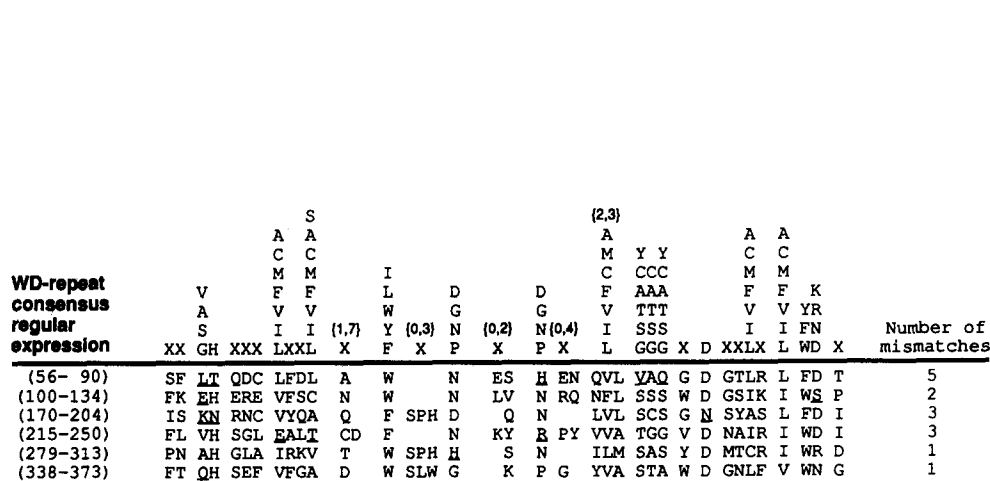


Figure 7. Alignment of the six WD repeats of *Peb1p* with the consensus WD sequence. The consensus sequence at the top is that given by Neer et al. (42); any one of the amino acids in a vertical column may occur at that position. X denotes any amino acid. The curly brackets above some symbols indicate the range over which that symbol may be repeated. The amino acids in *Peb1p* that do not match the consensus are underlined. Numbers in parentheses on the left indicate the first and last amino acid residues of that repeat.

tracellular distribution of thiolase (Fig. 5 B). Thiolase packaging appeared to be incomplete in JW86: a mixture of punctate and diffuse fluorescence was observed, indicating that some thiolase was packaged into peroxisomes but some was left in the cytosol. This conclusion was confirmed by digitonin permeabilization of strains JW86 and 88 (Fig. 6, C and D). The wild-type, untagged *PEB1* gene with 1,110-bp sequence of 5' upstream untranslated region was also cloned in a Yip plasmid and expressed at the normal level; the transformant showed a wild-type fluorescence pattern with anti-thiolase (data not illustrated). Thus epitope-tagged Peblp is functional, but it works less efficiently than the untagged protein.

Subcellular Location of Peblp

We made use of the fact that the tagged Peblp was functional and could be detected with the monoclonal antibody at its normal expression level to investigate the subcellular location of this protein. For this analysis, all cells were grown in glycerol and oleate medium to induce peroxisomes and three techniques were used.

Immunofluorescence. Immunofluorescence analysis of JW86 with the antibody 12CA5 showed weak, but distinct, punctate staining, indicating the organellar (peroxisomal?) location of Peblp (Fig. 9). In JW88, in which the triple-tagged Peblp was overexpressed, a mixed population of cells was seen. Some had stronger (as compared with JW86), distinct punctate staining with little cytosolic fluorescence; others had both punctate staining and cytosolic fluorescence. The *PEB1* knockout strain, JW75, was not fluorescent (Fig. 9), consistent with its lack of an epitope-tagged gene. These results suggest that at a normal expression level, Peblp is mainly organelle associated; overexpression of this protein results in increased association with organelles as well as some cytosol localization.

Digitonin Permeabilization: The intracellular location(s) of Peblp was further investigated by the selective permeabilization of spheroplasts with increasing concentrations of digitonin (Fig. 6). In JW86, the release pattern of tagged Peblp was essentially the same as that of catalase, strongly suggesting that this protein is located mostly or entirely in the peroxisomes in this strain. In JW88, the release pattern of Peblp upon digitonin clearly shows two plateaus. About half of the Peblp is released with the cytosolic proteins and the rest emerges with the peroxisomal proteins, thiolase and

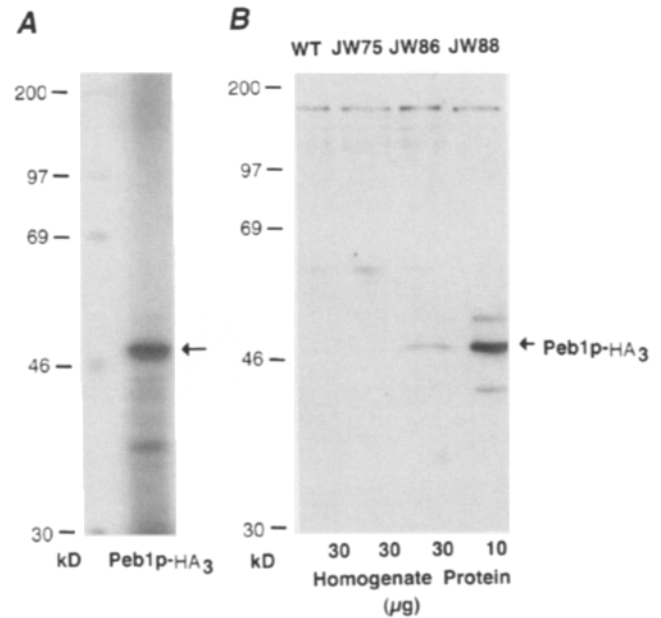


Figure 8. Expression of the triple-tagged Peblp. (A) In vitro expression of Peblp-HA₃ (arrow). Fluorographic detection. (B) In vivo expression of triple-tagged Peblp. Glass bead homogenates of cells grown in YPGO were separated by SDS-PAGE and immunoblotted with monoclonal antibody 12CA5 against the HA epitope. Chemiluminescence detection. Strain designations as in Fig. 5.

catalase. These data indicate a dual location of Peblp in JW88, partly in the cytosol, as indicated by the first plateau, and partly in peroxisomes, as indicated by the second plateau. This is consistent with the immunofluorescence data (Fig. 9) that showed both cytosolic and organelle localization in some cells.

Cell Fractionation. As a further test of Peblp's intracellular localization, classic cell fractionation was carried out on strain JW86, in which Peblp is produced at normal levels. A crude organelle fraction (25,000-g pellet), consisting mostly of mitochondria and peroxisomes, was prepared by differential centrifugation. It contained 32% of the Peblp, 13% of the thiolase, and 84% of the catalase activity of the post-nuclear supernatant, with the balance found in the 25,000-g supernatant (recoveries 80–100%). The reason that less Peblp

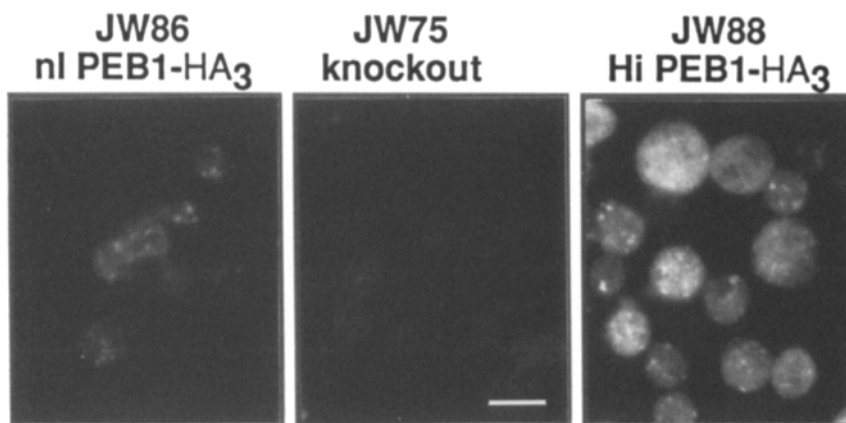


Figure 9 Immunofluorescence detection of triple-tagged Peblp with antibody 12CA5. Strain designations as in Fig. 5. Bar, 5 µm.

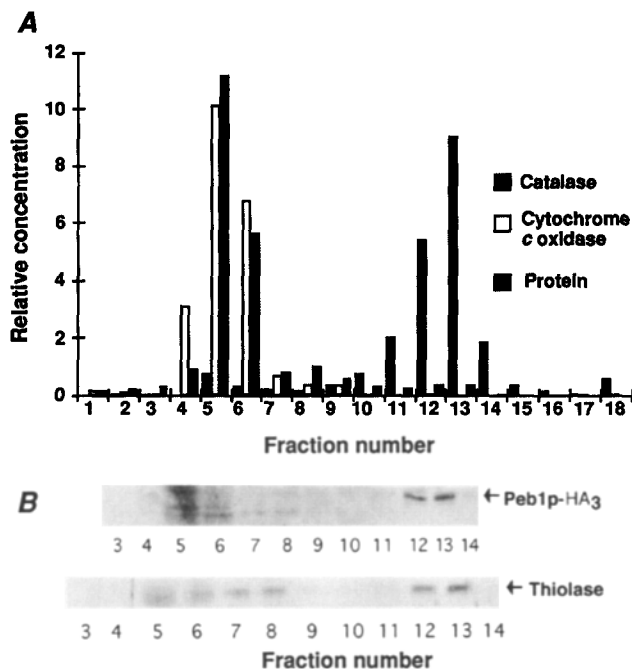


Figure 10. Nycodenz density gradient fractionation of JW86. (A) Distribution of catalase activity, cytochrome c oxidase activity and protein in the gradient. The ordinate represents relative concentrations as calculated according to de Duve (1967). The relative concentration equals the absolute concentration in each fraction divided by the concentration that would have been found if the enzyme was distributed uniformly through the gradient. (B) Distribution of triple-tagged Peblp and thiolase in fractions 3–14, detected by immunoblotting. 10 μ l of each fraction was separated by SDS-PAGE. Chemiluminescence was used for the detection.

was sedimentable than was found in peroxisomes by digitonin permeabilization will be discussed below.

The organelle fraction was subjected to equilibrium density gradient centrifugation in Nycodenz to purify the peroxisomes (Fig. 10). Of the Peblp-HA₃ detected in the gradient fractions, 70% was found with the peroxisomes in fractions 12 and 13 (total recovery on the gradient = 130% of the load). Small amounts of peroxisomal proteins, including catalase, thiolase and Peblp, were found in fractions near the top of the gradient, overlapping with the big peak of protein. Similar results are observed for thiolase and catalase in the fractionation of wild-type cells (not illustrated); this is due to the leakage of these enzymes from peroxisomes during pelleting and/or resuspension of the organelle fraction during the initial differential centrifugation. Qualitatively similar results on the presence of Peblp in peroxisomes were obtained by the classic cell fractionation of strain JW88 (78) and by the fractionation of a strain in which the thiolase gene was knocked out of wild-type JW68-3A and pBXNPEBI-HA₃ was expressed (Zhang, J. W., unpublished data).

Intra-peroxisomal Location of Peblp: Proteinase K Treatment

A proteinase K digestion experiment was carried out to test the location of Peblp in relation to the peroxisomal membrane. The experimental design was based on that of Hohfeld

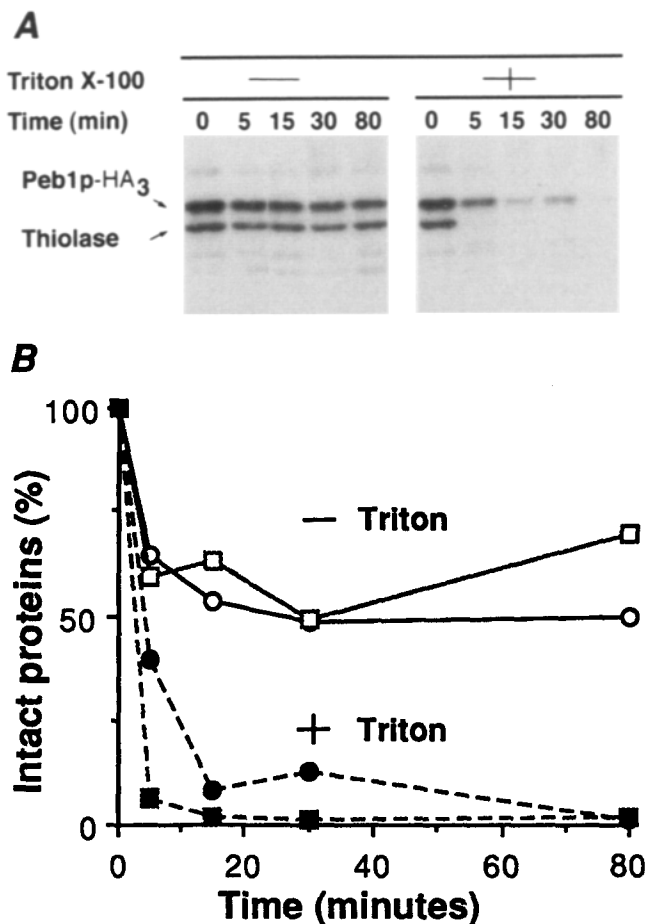


Figure 11. Accessibility of Peblp to proteinase K. An organelle fraction from JW88, consisting largely of peroxisomes and mitochondria, was treated with proteinase K coupled to agarose beads (25 mg/ml) for the times indicated, in the absence (-) and presence (+) of 0.1% Triton X-100 at room temperature. (A) Peblp-HA₃ and thiolase were detected by consecutive immunoblottings with monoclonal antibody 12CA5 and anti-thiolase. The sequence of lanes from one exposure has been reordered for clarity. (B) The amounts of intact Peblp-HA₃ (circles) and intact thiolase (squares) were quantitated and normalized to the undigested values.

et al. (24), who showed that Pas3p, a transmembrane peroxisomal protein with a large cytosolic domain, could be totally digested by proteinase K tethered to agarose beads under conditions where thiolase, a matrix protein within peroxisomes, was protected from digestion by the organelle membrane. We mixed the crude organelle fraction from JW88 with tethered proteinase K. Digestion was carried out for different lengths of time, in the absence and presence of 0.1% Triton X-100, after which the amount of Peblp remaining intact was determined by immunoblotting (Fig. 11). Because peroxisomes are fragile, and may lose matrix (content) proteins during the preparation of the organelle fraction, we controlled for this leakage by simultaneously analyzing the digestion of thiolase. In the absence of detergent, ~40% of thiolase was digested in the first 5 min, whereas the remaining 60% of the thiolase was not digested during the next 75 min (Fig. 11 B, open squares). In the presence of detergent, thiolase digestion was rapid and complete (closed squares).

These data indicate that ~60% of the control matrix protein, thiolase, remained within intact peroxisomes for the duration of the experiment when detergent was omitted.

The behavior of tagged Peblp was similar to that of thiolase. About 40% of Peblp was digested rapidly and ~60% was protected from digestion in the absence of detergent (*open circles*). Peblp was fully digested when detergent was added (*closed circles*). It took a little longer to complete the digestion of Peblp than to finish thiolase digestion, indicating that Peblp is a little less susceptible to this protease than is thiolase. The very similar behavior of Peblp and thiolase in this experiment suggests that the tagged Peblp in the organelle fraction is inside the peroxisomes together with thiolase.

Intra-peroxisomal Location of Peblp: Immunogold Labeling

The conclusion that Peblp is inside peroxisomes, protected by the peroxisomal membrane from external protease, was further tested by immunogold labeling of JW88 with antibody 12CA5. As shown in Fig. 12 *A*, the peroxisomes were labeled with gold particles, and the particles were mainly located over the matrix. This result indicates that Peblp is located in the peroxisomal matrix rather than in the membrane, and certainly not on the cytosolic face of the membrane. As expected, the mitochondria, recognizable by their cristae, were not labeled.

Further confirmation of the colocalization of Peblp with thiolase in the peroxisomal matrix was obtained by double-labeling immunoelectron microscopy. Peblp (large gold particles) and thiolase (small gold particles) are observed together in Fig. 12, *B* and *C*. Moreover, Peblp was shown to be within peroxisomes by double-labeling with rabbit antiserum 10-324, that recognizes several peroxisomal proteins (61) (Fig. 12, *D* and *E*).

Test of Possible Modification of Thiolase by Peblp

In view of the fact that Peblp is located inside peroxisomes, we considered the possibility that it might somehow modify thiolase during or after thiolase's entry into peroxisomes. As a first step in testing this, we compared the mobility in SDS-PAGE of thiolase before and after entry into peroxisomes with the mobility of thiolase from the strain lacking Peblp (Fig. 13). Thiolase synthesized *in vivo* in the *PEB1* knockout (lanes 1 and 2) had the same mobility as thiolase synthesized *in vivo* in wild-type yeast (lanes 3 and 4). The thiolase synthesized *in vivo* comigrated (lane 5) with thiolase expressed from the wild-type gene by *in vitro* transcription and translation (lanes 6 and 7). As a control, thiolase was expressed *in vitro* from the second methionine at residue 17; it migrated substantially faster (lane 8). Thus there is no indication for

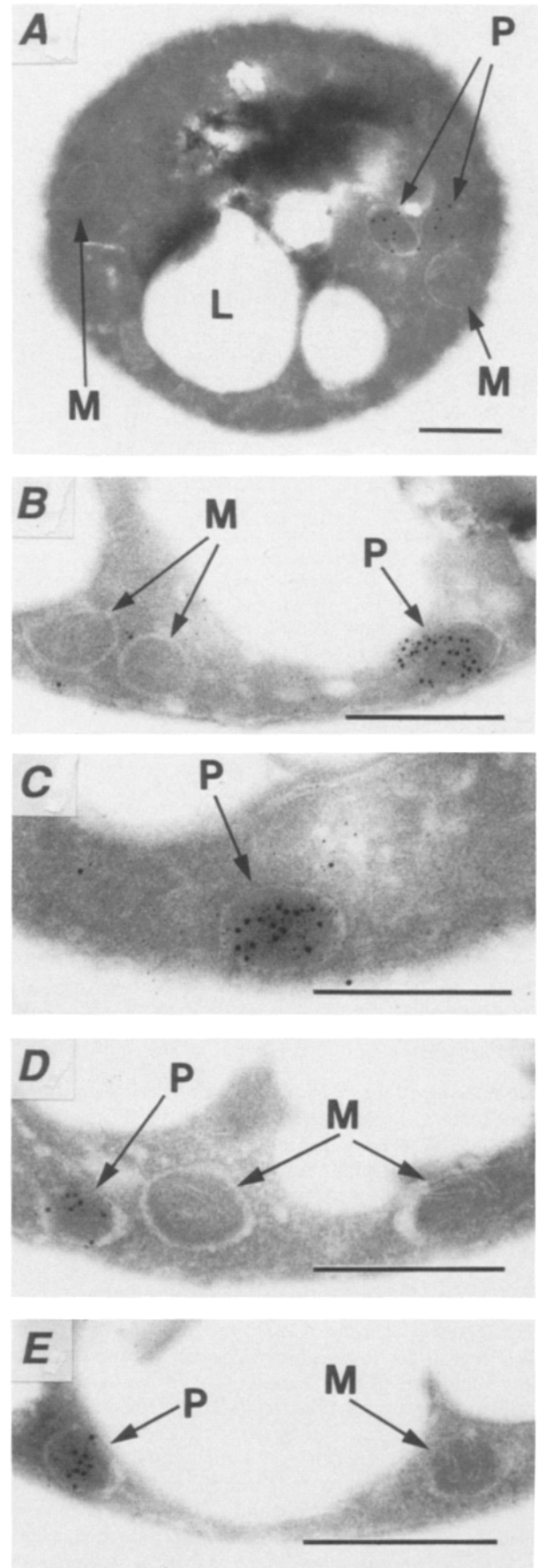


Figure 12. Immunoelectron microscopic detection of Peblp. JW88 cells were grown in YPGO. (*A*) Sections were incubated with monoclonal antibody 12CA5, followed by gold-conjugated protein A. (*B* and *C*) Sections were double-labeled with mouse monoclonal 12CA5 and rabbit anti-thiolase. Thereafter they were incubated with 20 nm gold coupled to goat anti-mouse IgG and with 5 nm gold coupled to goat anti-rabbit IgG. (*D* and *E*) Sections were double-labeled with mouse monoclonal 12CA5 (large gold) and rabbit antiserum 10-324 that reacts with several peroxisomal proteins (small gold). L, lipid droplet; M, mitochondrion; P, peroxisome. Bar, 0.5 μ m.

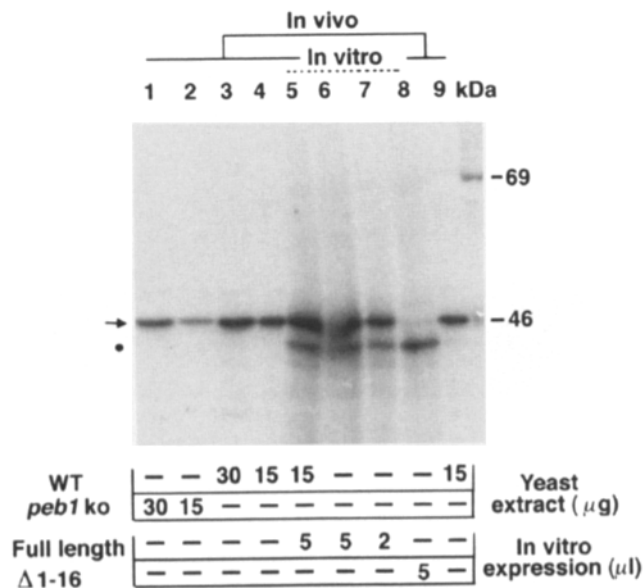


Figure 13. Thiolase synthesis in vivo and in vitro. The full-length thiolase gene (lanes 5–7) and the thiolase gene lacking codons for the first 16 amino acids (lane 8) were expressed in vitro, labeled with [³⁵S]methionine and [³⁵S]cysteine. The full-length product is indicated with an arrow. Some translation product that apparently began with the Met at residue 17 was routinely seen as a second product of the full-length gene (*dot*). Unlabeled glass bead homogenates of wild-type yeast (JW68-3A, lanes 3–5 and 9) or of the *PEB1* knockout (JW75, lanes 1 and 2) were subjected to SDS-PAGE and immunoblotted with rabbit anti-thiolase and [¹²⁵I]protein A. ³⁵S and ¹²⁵I on the nitrocellulose were detected with a PhosphorImager (Molecular Dynamics). The in vitro products were mixed with the yeast extract in lane 5. Control experiments demonstrated that the translation product abundance was too low to be detected by immunoblotting.

any proteolytic processing or other covalent modification of thiolase by *Peb1p*.

Discussion

We have cloned the *PEB1* (*PAS7*) gene by complementing the *peb1* mutant with a yeast genomic library. The authenticity of the *PEB1* gene was confirmed by gene disruption and genetic comparison of the knockout strain with the original mutant. This gene is not essential for growth on glucose, nor on glycerol, but is required for growth on oleate because it is necessary for the import of thiolase into peroxisomes.

Peb1p Is a Member of the WD Repeat Family

The sequence analysis of *Peb1p* revealed that it contains six WD repeats (or β -transducin repeats), putting this protein into a family of at least 27 other proteins with diverse intracellular locations and functions (42, 68). The conserved core of the repeat begins with Gly-His, ends with Trp-Asp (WD) and in between contains ~27 amino acids, many of which are hydrophobic and occur at conserved spatial positions. The core region is thought to fold into three β sheets, separated by turns. In addition to the core, the repeat contains a region of variable length, ranging from 6–94 amino acids, but commonly ~11 residues, for a total repeat length of

36–46 residues, generally. Proteins in this family contain from four to eight such repeats. The core regions are thought to associate with one another as dimers, or perhaps as multimers, and the exposed surfaces are thought to interact with other proteins (42). Proteins from this family are involved in remarkably varied cellular pathways, including signal transduction (β subunit of heterotrimeric G proteins, e.g., STE4), cell division (CDC4), gene regulation (TUP1), RNA processing (PRP4), and vesicular traffic (SEC13). Some members of this WD family interact with multiple partner proteins, none of which have structural features in common. A role in assembling protein complexes has been proposed (42).

Some proteins in the WD family have been found to be functionally related to members of another family of proteins containing tetratricopeptide repeats (TPR) (17). Proteins in the TPR family contain repeats of a degenerate 34-amino acid motif with conserved residue spacing; the TPR repeats are thought to interact with one another in a knob and hole configuration (17). Examples of functionally related pairs from the WD and TPR families are SSN6 (CYC8)/TUP1 that are involved in gene regulation, PRP4/PRP6 that are involved in RNA processing, and CDC20/CDC16 that are involved in cell division (for review see reference 17). One such pair of proteins, SSN6 (CYC8)/TUP1, has been shown to be physically associated with each other (74), and it has been suggested that other pairs of proteins from these two families might also interact physically (17). In this regard it is noteworthy that the recently cloned *PAS8* gene product in *Pichia pastoris* (40) and its homologue in *S. cerevisiae* (*Pas10p* [66]) contain seven and eight TPR motifs, respectively. This gene product is required for the import into peroxisomes of proteins containing the SKL targeting sequence but is not required for the import of thiolase. Thus, this function is complementary with that of *Peb1p*. It would be interesting to test the possibility that *Pas10p* and *Peb1p* might interact with one another.

Peb1p Is an Intra-peroxisomal Protein

Epitope tagging was used to study the subcellular location of *Peb1p*. The triple-tagged *PEB1* gene product was found associated with peroxisomes by immunofluorescence (Fig. 9), digitonin permeabilization (Fig. 6), equilibrium density gradient centrifugation (Fig. 10) and immunoelectron microscopy (Fig. 12). The percentage of *Peb1p* in peroxisomes depended on the expression level. When the tagged *PEB1* gene was integrated into the genome and expressed under the control of its own promoter, *Peb1p* appeared to be exclusively associated with peroxisomes according to digitonin permeabilization. This conclusion is based on the remarkable similarity in the release of *Peb1p* (plus symbols) and of catalase (circles) as a function of digitonin concentration (Fig. 6 C).

In contrast, less than a third of *Peb1p* was found in peroxisomes by classical cell fractionation (Fig. 10). We and others have repeatedly observed substantial and variable leakage of peroxisomal matrix proteins during homogenization and cell fractionation, especially with *S. cerevisiae* (1, 10, 11, 24, 37, 39, 61, 62, 66). For example, in fractionation of wild-type cells, ~15–49% of thiolase, 15–35% of acyl-CoA oxidase, ~15% of the multifunctional β -oxidation protein, and 10–70% of catalase (in some experiments this includes a variable and unknown amount of the cytosolic isozyme, catalase T) are found in the post-organelle supernatant (10,

11, 24, 37, 39, 61, 66), despite the fact that these enzymes are thought to be entirely in peroxisomes in vivo. The digitonin method is far more gentle because it avoids mechanical disruption of organelle membranes. Thiolase, catalase, and acyl-CoA oxidase are all found at least 93% within peroxisomes in wild-type *S. cerevisiae* by digitonin permeabilization (80). Therefore, we believe that digitonin permeabilization is more reliable than classic cell fractionation for the quantitative estimation of how much of an enzyme is in yeast peroxisomes. In the present experiments, Peblp leaked more readily than catalase from damaged peroxisomes. Similar unequal leakage of enzymes occurs with purified rat liver peroxisomes (1). Significantly, Peblp, at its normal expression level, behaved during digitonin titration, within experimental error, like catalase.

Overexpression of Peblp in strain JW88 resulted in this protein being located in the cytosol as well as in peroxisomes. Peblp release by digitonin was biphasic with about half emerging from spheroplasts with the cytosolic marker and the other half with catalase (Fig. 6 D). The appearance of Peblp in the cytosol in JW88 may be the result of saturation of the peroxisomal import machinery for the overexpressed Peblp.

The immunofluorescence data are consistent with the digitonin data. A non-homogeneous staining pattern was observed for Peblp in JW88, with some cells demonstrating mainly punctate fluorescence and other cells having both particles and substantial cytosolic fluorescence (Fig. 9). This probably reflects different expression levels of Peblp among the cells, due to variance in the copy number of the episomal plasmid among the cells (that is inherent to the use of this vector). In contrast, cytosolic fluorescence was not seen at the normal expression level.

We expected that Peblp associated with peroxisomes would be exposed to the cytosol if it were to serve as a receptor to bring newly synthesized thiolase to peroxisomes, as hypothesized in the Introduction. Surprisingly, the accessibility of Peblp to externally added proteinase K was indistinguishable from that of thiolase, a protein known to be localized within peroxisomes, which served as a control (Fig. 11). These data indicated that Peblp was also inside peroxisomes. This was unequivocally confirmed by immunogold labeling (Fig. 12 A) and by immunogold double-labeling with antibodies against other peroxisomal proteins (Fig. 12, B-E). The data do not exclude the possibility that some Peblp might be associated with the inner aspect of the peroxisomal membrane.

As the triple-tagging of Peblp did not introduce an SKL-type sequence at the COOH terminus of the protein, it is unlikely that the peroxisomal location of the tagged Peblp is due to the tagging. Therefore, the targeting information in the tagged protein must reside elsewhere in the Peblp sequence. As shown in Fig. 7, almost the whole Peblp, except for the NH₂-terminal 55 amino acids, consists of six WD repeats. Comparison of the NH₂-terminal sequence with that of yeast or mammalian peroxisomal thiolase revealed no obvious homology. Further experiments are required to determine the location of the targeting information in Peblp.

Our conclusion that Peblp (Pas7p) is an intra-peroxisomal protein differs from that of Marzioch et al. (37), who concluded that this is mainly a cytosolic protein, 5–15% of which associates in their model with the external surface of

the peroxisome. This may be due to the different methods employed. Marzioch et al. only studied cells in which Pas7p was overexpressed with the CUP1 promoter. Secondly, they used classical cell fractionation and not the gentler digitonin permeabilization method. Rather similar results were obtained by the two groups with cell fractionation: they reported that 5–15% of the Pas7p in a homogenate sedimented with peroxisomes; we observed that 32% of the protein sedimented with the crude organelle fraction, of which ~70% (i.e., 22% total) was subsequently found in peroxisomes by density gradient centrifugation. Thirdly, Marzioch et al. attached the myc epitope tag at the NH₂ terminus whereas we attached the HA tag to the COOH terminus; conceivably the NH₂-terminal tag might interfere with import. They did not test whether the Peblp (Pas7p) was inside peroxisomes or attached to the outside surface; the 5–15% of myc-Pas7p might have been inside peroxisomes. So there is no experimental disagreement on this point. The finding that Peblp-HA₃ is in peroxisomes even without thiolase expression whereas myc-Pas7p was entirely cytosolic without thiolase expression might reflect the difference in tags or perhaps some difference in strains.

Epitope Tagging Affects Peblp Function

When the triple-tagged Peblp was expressed at normal levels under its native promoter, it only partially rescued thiolase packaging and growth on oleate (Figs. 5 and 6). The untagged, wild-type copy of the gene expressed in the same vector fully restored the function of the *pebl* null mutant (data not shown). These results indicate that the addition of the HA epitope on the COOH terminus of Peblp decreased its function. This phenomenon has been seen in other proteins with epitope tagging (9). The reduced function could be fully overcome by increasing the expression level (Figs. 5 and 6).

The Function of Peblp in Peroxisome Biogenesis

Peblp (Pas7p) function is required for the import into peroxisomes of thiolase (which contains an amino-terminal type 2 targeting sequence) but not for the import of catalase or luciferase (see Results) or for the multifunctional β -oxidation protein or several other proteins (37) that contain a type 1 carboxy-terminal tripeptide targeting sequence. This phenotype suggested that Peblp might be a receptor that would cause newly synthesized thiolase to become docked on the outside of peroxisomes, ready to be translocated inside. Since the data indicate that Peblp is located inside the peroxisomes, this role for Peblp is not feasible. This function may be fulfilled by some other protein, yet to be identified. In any event, other role(s) for Peblp have to be considered.

One possibility that we considered is that Peblp might cleave the NH₂-terminal peroxisomal targeting sequence from thiolase after it enters the peroxisome. Such proteolytic processing is known to occur for the rat (43, 60) and human (53) peroxisomal thiolases. The rat thiolases are cleaved after 36 amino acids (thiolase A) or after 26 amino acids (thiolase B) (60). *S. cerevisiae* thiolase is likewise targeted to peroxisomes by its first 16 amino acids (that show substantial sequence similarity to the rat presequence) (10, 16), but these 16 amino acids are reported not to be cleaved after import

16 amino acids are reported not to be cleaved after import (16). Purified *S. cerevisiae* thiolase was found to lack its first six amino acids; it was considered uncertain whether this was due to physiological processing or to a purification artifact (38). In view of the possibility of yeast strain differences, we reinvestigated the question, and found no detectable proteolytic processing of thiolase (Fig. 13). Thiolase synthesized *in vivo* in the *PEB1* knockout (which remains in the cytosol) had the same mobility as peroxisomal thiolase made in wild-type cells, so the apparent size of thiolase is unaffected by the presence or absence of Peb1p in the cell. It remains a theoretical possibility that Peb1p might modify thiolase covalently in some fashion that does not appreciably affect its mobility in SDS-PAGE. Were this the case, loss of Peb1p function might interfere with thiolase import, in a fashion analogous to the mitochondrial protein import defect caused by mutations in the mitochondrial processing protease (*mas1*, *mas2/mif2*) (25, 44, 77).

Another possibility for the function of Peb1p is that it might work as an intra-organellar receptor for thiolase import (Fig. 14). Perhaps, thiolase is first recognized by an extra-peroxisomal receptor (yet to be identified) and inserted through the peroxisome membrane. Peb1p within the peroxisomes could then bind the thiolase peptide and, probably with the participation of other translocation factors, "pull" the thiolase into the peroxisomes. This proposed role of Peb1p in thiolase import resembles somewhat that of the mitochondrial Hsp70 (*SSCI* gene product), which binds to newly synthesized proteins as they enter mitochondria (27). Mitochondrial Hsp70 has a function in mitochondrial protein translocation distinct from its unfolding function (14).

This model is consistent with our finding Peb1p in peroxisomes even when no thiolase is expressed (see Results).

Perhaps Peb1p contributes to the folding of thiolase within peroxisomes. Such a role, too, could lead to a defect in thiolase import in *peb1* mutants. By way of precedent, the 70-kD heat shock protein in the ER, BiP (the product of the *KAR2* gene), is required for the translocation of secretory proteins into the ER *in vivo* (71). Recent evidence indicates that BiP, like mitochondrial Hsp70, functions at two distinct stages in the ER translocation cycle (50). Further experiments are required to explore the possibility of a specific binding of Peb1p with thiolase and to elucidate Peb1p's role in thiolase import into peroxisomes.

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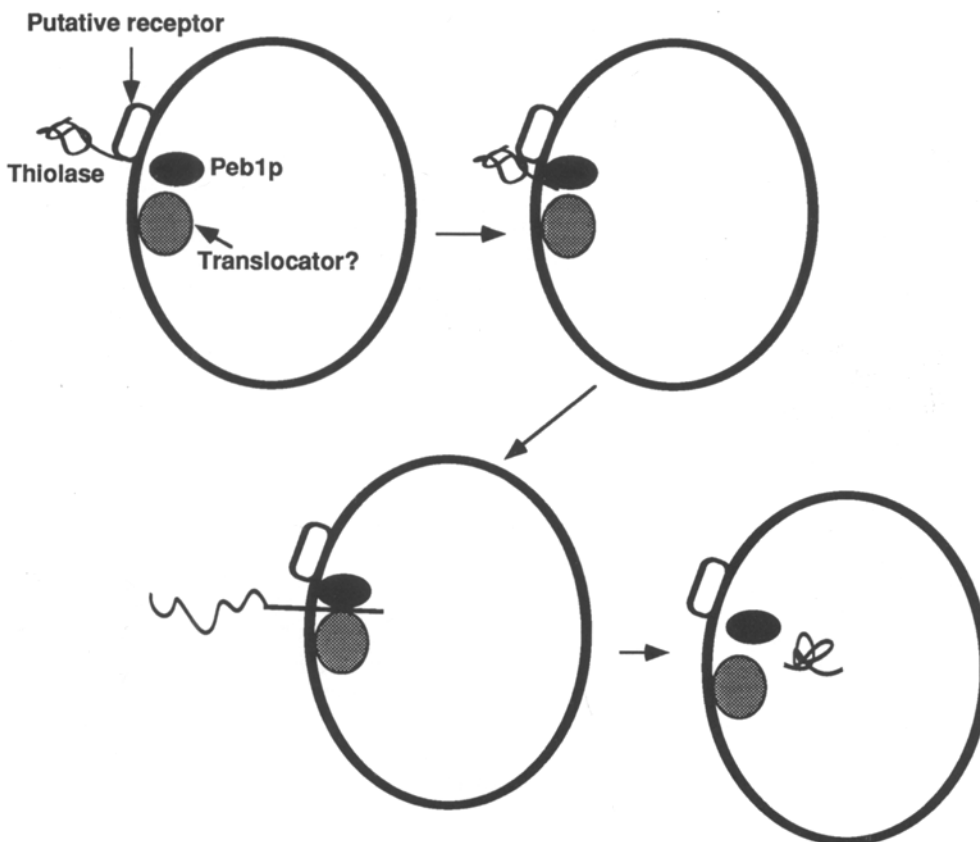


Figure 14. Possible role of Peb1p in thiolase import: speculative model. A receptor binds the thiolase NH₂-terminal targeting sequence after it is translated and inserts it through the peroxisomal membrane. Peb1p inside the peroxisome recognizes and binds to the thiolase NH₂-terminal sequence. Thiolase is then pulled inside, perhaps with the participation of additional translocation factors.

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