**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* (*pasT*) 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 *PEB1* 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 *PEB1* 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 *PEB1* 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 proteins. 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-
zomal 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 (pebl) 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-I does not package thiolase, but the import of catalase and acyl-CoA oxidase is normal; pebl-5-I 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 pasl0 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 much lower digitonin concentrations (300–600 µg/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-I 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 PEB1 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 PEB1 gene. We found that PEB1 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 PEB1 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 isoform) but defective catalase T (the cytosolic isoform) (cut-l). 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 PEB1 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 x 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.

Table I. Yeast Strains Used in This Study

| Name | Genotype | Sources |
|------|----------|---------|
| JW683A | MATa, ura3-1, trp1-1, arg4, clt-1 | Reference 79 |
| pebl-1 | MATa, pebl-1, ura3-1, trp1-1, clt-1 | Reference 79 |
| pebl-1-R1 | MATa, pebl-1, ura3-1, trp1-1, clt-1 | (m6-D1) |
| JW75* | MATa, ura3-1, trp1-1, arg4, clt-1, pep1::TRP1 | This study |
| JW86* | MATa, ura3-1, trp1-1, arg4, clt-1, pep1::TRP1 | This study |
| JW88* | MATa, ura3-1, trp1-1, arg4, clt-1, pep1::TRP1 | This study |
| [pBXNPEB1-HA] | | |

Abbreviations used in this paper: HA, hemagglutinin; ORF, open reading frame; pas, peroxisome assembly; pebl, peroxisome biogenesis; Peblp, protein encoded by the PEB1 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.

* Isogenic with JW683A.
Table II. Plasmids Used in This Study

| Name               | Characteristics                                      | Source         |
|--------------------|------------------------------------------------------|----------------|
| pR1                | 10-kb fragment complementing the pebl-1 mutant. Came from the genomic library (reference 47). URA3. Ycp50 based. | This study     |
| pR2                | 3-kb fragment complementing pebl-1. Cloned in pRS314 (see below)                                      | This study     |
| pRSPEBI            | PEB1 ORF including 53-bp upstream untranslated sequence cloned in pRS314. T3 promoter.                 | This study     |
| pRSPEBI-HA3        | PEB1 ORF including 53-bp upstream untranslated sequence tagged with three copies of the HA epitope cloned in pRS306. T3 promoter. | This study     |
| YipPEBI-HA3        | Triple-tagged PEB1 including 1110-bp upstream untranslated sequence cloned in pRS306.                    | This study     |
| pBXNPEBI-HA3       | Triple-tagged PEB1 ORF including 53-bp upstream untranslated sequence cloned in pAB23BXN. GAPDH promoter. 2µ | This study     |
| pJW17              | pebl::TRP1. The Bgl2 fragment of the PEB1 gene was replaced with the TRP1 gene. Based on pR2. See Fig. 2 D. | This study     |
| pRB58              | A 12-kb genomic fragment containing P0TI (thiolase gene) and SUC2, 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 Δ1-16  | The thiolase ORF with deletion of sequence encoding the first 16 amino acid residues, cloned in pRS316. T3 promoter. | This study     |
| pRS314             | TRPI. CEN4.                                           | Reference 55   |
| pRS306             | URA3. Yip.                                            | Reference 55   |
| pRS316             | URA3. CEN4.                                           | Reference 55   |
| pAB23BXN           | URA3. GAPDH promoter. 2µ                              | 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 pRSPEBI, pRSPEBI-HA3, pRSThiolase, and pRSThiolaseΔ1-16 were transcribed with T3 RNA polymerase after linearization. RNA was extracted with phenol/chloroform, ethanol-purified, 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 [3H]methylated markers (CPA7561; American 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) (YPYDVPDYA), 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 wild-type 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 GAC 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 COOH terminus of the PEB1 gene. The intended oligonucleotide sequence was 5'TCA TTA GCC G(T)TA ATC AGG AAC GTC ATA CGG GTA GGA TCC TCC ATA GTC CGG TAC ATC ATA GGG ATA GCC 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.

Proteinase K Treatment of Organelles

A crude organelle fraction was subjected to gentle proteolysis as described by Hoitiek 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-µl 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 µl 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 Vansell and Pelak (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 peroxins and peroxins (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 1:150 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 × 10^5 cpm/ml, Fig. 13). Immunoblot data were quantitated with a Personal Den- sitometer (Molecular Dynamics, Sunnyvale, CA) (80). The DNA recombi-
nation 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 Cor-

pation (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 pebl-I 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, pebl-I-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 pebl-I-R1 (containing plas-
mid) 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 re-
trieved from this transformant and found to contain a 10-kb
insert. Plasmid pR1 was transformed back into pebl-I, where
it restored thiolase packaging and peroxisomal function to
the mutant, as seen by growth on YNO medium and im-
munofluorescence 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
pebl-I and tested for their ability to complement the pebl
mutation. The smallest fragment with the ability to restore
the growth of the mutant pebl-I on a YNO plate was a 3-kb

Figure 1. Functional complementation of the pebl-I mutant. (A) The growth on oleic
acid (YNO plate) of pebl-I, pebl-I that had regained the ability to grow on YNO af-
ter transformation with a plasmid from a genomic library (pebl-I [pR1]), and wild
type (JW68-3A). (B) Immunofluorescence with anti-thiolase of cells grown in YPGO
medium to induce peroxisomes (see Methods). Bar, 5 #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 = PAST (37).

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**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 (YN0 Growth) and immunofluorescence with anti-thiolase (Punctate fluorescence). 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.
Disruption of this gene was confirmed by PCR amplification.

Deletion of the Chromosomal Copy of PEB1:

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

Like the original pebl-I 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 PEB1 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-I, 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 PEB1 knockout under the control of the POX1 promoter (72) and found in peroxisomes by immunofluorescence (data not shown).

The PEB1 knockout strain was crossed with the original pebl-I 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 PEB1 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 PEB1 ORF by means of PCR amplification. Due to a dropped base in a 90-bp oligonucleotide, the third epitope was imperfect (see Materials...
and Methods). The triple-tagged PEB1 ORF (termed PEB1-HA3) was expressed in vitro (Fig. 8 A) and a band of ~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 PEB1 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 PEB1 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 ~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 PEB1 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 PEB1 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 Peblp 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 Peblp 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 Peblp 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 \textit{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 Pebp is functional, but it works less efficiently than the untagged protein.

\textbf{Subcellular Location of Pebp}

We made use of the fact that the tagged Pebp 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.

\textit{Immunofluorescence.} Immunofluorescence analysis of JW86 with the antibody 12CA5 showed weak, but distinct, punctate staining, indicating the organellar (peroxisomal?) location of Pebp (Fig. 9). In JW88, in which the triple-tagged Pebp 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 \textit{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, Pebp is mainly organelle associated; overexpression of this protein results in increased association with organelles as well as some cytosol localization.

\textit{Digitonin Permeabilization:} The intracellular location(s) of Pebp was further investigated by the selective permeabilization of spheroplasts with increasing concentrations of digitonin (Fig. 6). In JW86, the release pattern of tagged Pebp 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 Pebp upon digitonin clearly shows two plateaus. About half of the Pebp is released with the cytosolic proteins and the rest emerges with the peroxisomal proteins, thiolase and catalase. These data indicate a dual location of Pebp 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.

\textit{Cell Fractionation.} As a further test of Pebp's intracellular localization, classic cell fractionation was carried out on strain JW86, in which Pebp 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 Pebp, 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 Pebp

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8}
\caption{Expression of the triple-tagged Pebp. (A) In vitro expression of Pebp-HA\textsubscript{3} (arrow). Fluorographic detection. (B) In vivo expression of triple-tagged Pebp. 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.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9}
\caption{Immunofluorescence detection of triple-tagged Pebp with antibody 12CA5. Strain designations as in Fig. 5. Bar, 5 \mu m.}
\end{figure}
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 Peb1p-HA$_3$ 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 Peb1p, 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 Peb1p 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 pBXNPEB1-HA$_3$ was expressed (Zhang, J. W., unpublished data).

**Intra-peroxisomal Location of Peb1p: Proteinase K Treatment**

A proteinase K digestion experiment was carried out to test the location of Peb1p in relation to the peroxisomal membrane. The experimental design was based on that of Hohfeld 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 Peb1p 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 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
any proteolytic processing or other covalent modification of thiolase by Peblp.

Discussion

We have cloned the PEB1 (PAS7) gene by complementing the pebl 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.

Peblp Is a Member of the WD Repeat Family

The sequence analysis of Peblp 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 (Pasl0p [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 Peblp. It would be interesting to test the possibility that Pasl0p and Peblp might interact with one another.

Peblp Is an Intra-peroxisomal Protein

Epitope tagging was used to study the subcellular location of Peblp. 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 Peblp 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, Peblp appeared to be exclusively associated with peroxisomes according to digitonin permeabilization. This conclusion is based on the remarkable similarity in the release of Peblp (plus symbols) and of catalase (circles) as a function of digitonin concentration (Fig. 6 C).

In contrast, less than a third of Peblp 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. 6D). 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. 12A) 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-HA, 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 \textit{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 \textit{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 Peblp in the cell. It remains a theoretical possibility that Peblp might modify thiolase covalently in some fashion that does not appreciably affect its mobility in SDS-PAGE. Were this the case, loss of Peblp function might interfere with thiolase import, in a fashion analogous to the mitochondrial protein import defect caused by mutations in the mitochondrial processing protease (\textit{masl, mas2/mif2}) (25, 44, 77).

Another possibility for the function of Peblp 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. Peblp 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 Peblp in thiolase import resembles somewhat that of the mitochondrial Hsp70 (\textit{SSC1} 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 Peblp in peroxisomes even when no thiolase is expressed (see Results).

Perhaps Peblp contributes to the folding of thiolase within peroxisomes. Such a role, too, could lead to a defect in thiolase import in pebl mutants. By way of precedent, the 70-kD heat shock protein in the ER, BiP (the product of the \textit{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 Peblp with thiolase and to elucidate Peblp's role in thiolase import into peroxisomes.

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Figure 14. Possible role of Peblp 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. Peblp 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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