Peb1p (Pas7p) Is an Intraperoxisomal Receptor for the NH_2-terminal, Type 2, Peroxisomal Targeting Sequence of Thiolase: Peb1p Itself Is Targeted to Peroxisomes by an NH_2-terminal Peptide

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Abstract. *Peb1* is a peroxisome biogenesis mutant isolated in *Saccharomyces cerevisiae* that is selectively defective in the import of thiolase into peroxisomes but has a normal ability to package catalase, luciferase and acyl-CoA oxidase (Zhang, J. W., C. Luckey, and P. B. Lazarow. 1993. *Mol. Biol. Cell.* 4:1351–1359). Thiolase differs from these other peroxisomal proteins in that it is targeted by an NH_2-terminal, 16–amino acid peroxisomal targeting sequence type 2 (PTS 2). This phenotype suggests that the *PEB1* protein might function as a receptor for the PTS2. The *PEB1* gene has been cloned by functional complementation. It encodes a 42,320-D, hydrophilic protein with no predicted transmembrane segment. It contains six WD repeats that comprise the entire protein except for the first 55 amino acids. Peb1p was tagged with hemagglutinin epitopes and determined to be exclusively within peroxisomes by digitonin permeabilization, immunofluorescence, protease protection and immuno-electron microscopy (Zhang, J. W., and P. B. Lazarow. 1995. *J. Cell Biol.* 129:65–80). Peb1p is identical to Pas7p (Marzioch, M., R. Erdmann, M. Veenhuis, and W.-H. Kunau. 1994. *EMBO J.* 13: 4908–4917). We have now tested whether Peb1p interacts with the PTS2 of thiolase. With the two-hybrid assay, we observed a strong interaction between Peb1p and thiolase that was abolished by deleting the first 16 amino acids of thiolase. An oligopeptide consisting of the first 16 amino acids of thiolase was sufficient for the affinity binding of Peb1p. Binding was reduced by the replacement of leucine with arginine at residue five, a change that is known to reduce thiolase targeting in vivo. Finally, a thiolase–Peb1p complex was isolated by immunoprecipitation. To investigate the topogenesis of Peb1p, its first 56–amino acid residues were fused in front of truncated thiolase lacking the NH_2-terminal 16–amino acid PTS2. The fusion protein was expressed in a thiolase knockout strain. Equilibrium density centrifugation and immunofluorescence indicated that the fusion protein was located in peroxisomes. Deletion of residues 6–55 from native Peb1p resulted in a cytosolic location and the loss of function. Thus the NH_2-terminal 56–amino acid residues of Peb1p are necessary and sufficient for peroxisomal targeting. Peb1p is found in peroxisomes whether thiolase is expressed or not. These results suggest that Peb1p (Pas7p) is an intraperoxisomal receptor for the type 2 peroxisomal targeting signal.

Analysis of peroxisome biogenesis (*peb*)[1] mutants suggest that the import of newly synthesized proteins into peroxisomes involves a branched pathway (34, 51). The branches in this pathway are hypothesized to involve receptors for peroxisomal targeting sequences (PTSs) and the common steps in the import pathway presumably include components of the translocation machinery.

As is now well known, there are at least three types of targeting sequences for peroxisomal matrix proteins (proteins inside the organelle). A carboxy-terminal tripeptide, ser-lys-leu, or conservative variants of this sequence, directs diverse proteins to peroxisomes in animals, plants and yeasts (PTS1) (17, 18, 23). An amino-terminal oligopeptide consisting of 11–16 amino acids is necessary and sufficient for the targeting of thiolase to peroxisomes in mammals (32, 44) and *Saccharomyces cerevisiae* (9, 16). The *S. cerevisiae* sequence, MSQ**RL**QSIKD**HL**VLSA, contains two pairs of amino acids, RL and HL, that are required for the topogenesis and are conserved in the mammalian thiolases. This PTS2 is also found in watermelon

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1. *Abbreviations used in this paper*: HA, hemagglutinin epitope; *pas*, peroxisome assembly; *peb*, peroxisome biogenesis; Peb1p, protein encoded by the *PEB1* gene; PTS, peroxisomal targeting sequence.

malate dehydrogenase (14), amine oxidase in *Hansenula polymorpha* (11), *Perlp* in *Hansenula polymorpha* (47), and protein(s) in trypanosome microbodies (4). Still other peroxisomal proteins contain redundant internal targeting sequences; these include *Candida tropicalis* acyl-CoA oxidase (21, 42) and *S. cerevisiae* catalase A (24).

Mutants in the import of peroxisomal proteins have been isolated from several species (for reviews see 25, 26, 43). Many of them appear to be incapable of importing any matrix proteins into their peroxisomes, regardless of the type of PTS. These data imply that there are common components in the pathway for importing proteins with the various PTSs. These could include chaperones as well as membrane translocation proteins.

Mutants selectively defective in the import of peroxisomal proteins with a carboxyl-terminal PTS1 have been identified in yeasts and humans (29, 30, 46, 51). Cells carrying these mutations cannot import proteins targeted by a PTS1 into their peroxisomes, but do package normally the peroxisomal proteins bearing other PTSs. The corresponding genes have been cloned and characterized: Pas8p in *Pichia pastoris* (29), Pas10p in *S. cerevisiae* (46), and PXR1 (8) which is identical to PTS1 receptor (13, 48) in humans. *P. pastoris* Pas8p and the human protein bind specifically to a peptide ending with SKL but not to control peptides (8, 13, 29, 48). *S. cerevisiae* Pas10p and the human homologue have been shown to interact with proteins ending with SKL by means of the two hybrid assay (5, 13). Thus, these proteins are thought to be the PTS1 receptor.

S. cerevisiae mutant *peb1* (allelic with *pas7*) is defective in importing thiolase into peroxisomes but are competent to import most other peroxisomal proteins including those with PTS1s as well as acyl-CoA oxidase (28, 49, 51). The observed phenotype suggests that the gene product Peb1p (Pas7p) may be a receptor for the PTS2. We (50) and Marzioch et al. (28) have cloned the PEB1/PAS7 gene by functional complementation. It encodes a 42,320-D, hydrophilic protein with no predicted transmembrane segment. The protein contains six WD repeats, a motif that has been identified in 27 proteins involved in diverse cellular functions (31). The conserved core of the motif begins with Gly-His and after ~27 amino acids, ends with Trp-Asp (WD). Each repeat is thought to fold into three β sheets, separated by turns, and the repeats are believed to associate with one another as dimers or perhaps as multimers. The six WD motifs comprise the large majority of the PEB1 protein, with the exception of the first 55 amino acids.

We tagged the PEB1 protein at its carboxy terminus with three copies of the hemagglutinin (HA) epitope and demonstrated that the tagged protein (Peb1p-HA_3) was functional (50). Peb1p-HA_3 was determined to be associated with peroxisomes by immunofluorescence and equilibrium density centrifugation. When expressed at the wild type level under the control of its own promoter from a single-copy integrating plasmid, all of the Peb1p-HA_3 was found in peroxisomes (within experimental error) as assessed quantitatively by progressive permeabilization of the plasma membrane and intracellular membranes with increasing concentrations of digitonin. We observed that digitonin permeabilization is far gentler, and therefore more reliable for determining intracellular protein distributions, than classical cell homogenization followed by differential and equilibrium density centrifugations. Moreover,

Table I. Plasmids Used in This Study

Name	Construction	Source
YipPEB1-HA_3	*PEB1* tagged with three copies of the HA epitope under the control of its own promoter in an integrating plasmid, pRS306, which has a *URA3* marker.	(50)
YcpPEB1-HA_3	Triple-tagged *PEB1* under the control of its own promoter in a centromere plasmid, pRS315, containing a *LEU2* marker.	This study
pBXNPEB1-HA_3	Triple-tagged *PEB1* under the control of the GAPDH promoter in a multicopy 2μ plasmid.	(50)
YipPEB1Δ-HA_3	Identical to YipPEB1-HA_3 except that the nucleotides encoding amino acids 6–55 of Peb1p were deleted by PCR.	This study
YepPeb1$p_{(1-56)}$Thiolase$_{(17-417)}$	The part of the *PEB1* gene encoding amino acids 1–56 of the peb1p fused in frame with the part of the *POT1* gene encoding amino acids 17–417 of thiolase, under the control of the *PEB1* promoter in a multicopy, 2μ plasmid, Yep352.	This study
pPC97	The DNA binding domain (DB) of the *GAL4* protein in a centromere plasmid.	(6)
pGAL4 (DB)-PEB1	The EcoRV-Sac1 fragment of the *PEB1* gene (encoding residues 4–375) cloned in pPC97 after the *GAL4* DNA binding domain.	This study
pPC86	The transactivation domain (TA) of the *GAL4* protein in a centromere plasmid.	(6)
pGAL4 (TA)-POT1	The full length thiolase gene, *POT1*, cloned in pPC86 after the *GAL4* transactivation domain.	This study
pGAL4 (TA)-POT1Δ	Identical to pGAL4 (TA)-POT1 except that the nucleotides encoding the first 16 amino acids of thiolase were deleted.	This study
pGAL4 (TA)-Luciferase	The full length luciferase gene cloned in pPC86 after the *GAL4* transactivation domain.	This study
pJW30	The 16-bp EcoR1-Hind3 fragment of the thiolase gene (*POT1*) was replaced with the *TRP1* gene. Used for thiolase disruption to construct strain JW69.	This study
pJW130	The 16-bp EcoR1-Hind3 fragment of the thiolase gene was replaced with the *URA3* gene. Used for thiolase disruption to construct strain JW142.	This study
pRS306	Integrating plasmid. *URA3*.	(41)
pRS315	Centromere plasmid. *LEU2*.	(41)
Yep352	2μ *URA3*.	(19)

Table II. Yeast Strain Used in This Study

Name	Genotype and/or description	Source
JW68-3A (Wild type)	*MATα, ura3-1, trp1-1, arg4, ctt1-1*	(49)
JW69*	*MATα, ura3-1, trp1-1, arg4, ctt1-1, pot1::TRP1*	This study
JW75*	*MATα, ura3-1, trp1-1, arg4, ctt1-1, pot1::TRP1*	(50)
JW86*	JW 75 transformed with plasmid YipPEB1-HA_3	(50)
JW88*	JW 75 transformed with plasmid pBXNPEB1-HA_3	(50)
2m 1-A4	*MATα, peb4-1, leu2-3,112, ura3-1, trp1-1, arg4, ctt1-1*	(49)
JW89	2m1-A4 transformed with plasmid pBXNPEB1-HA_3	This study
JW142*	*MATa, ura3-1, trp1-1, arg4, ctt1-1, peb1::TRP1, pot1::URA3*	This study
JW142a‡	*MATa, ura3-1, trp1-1, arg4, leu2, his4, peb1::TRP1, pot1::URA3*	This study
JW161	JW 142a transformed with plasmid YcpPEB1-HA_3	This study
JW162*	JW75 transformed with plasmid YipPEB1Δ-HA_3	This study
JW146*	JW69 transformed with plasmid YepPeb1$p_{(1-56)}$ Thiolase$_{(17-417)}$	This study
SFY526	*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can^r, gal4-542, gal80-538, URA3::GAL1-LacZ*	(1)
W303a	*MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112*	(36)

*Isogenic with JW68-3A.
‡It is unknown whether JW142a is *CTT1* or *ctt1-1*.

protease protection studies demonstrated that Peb1p-HA_3 is inside the peroxisome, in the matrix space, together with thiolase. This was confirmed by immuno-electron microscopy (50).

These observations suggested the hypothesis that Peb1p functions as an intra-peroxisomal receptor during the import of thiolase into peroxisomes (50). If this is true, we would expect that Peb1p would bind specifically to the NH_2-terminal PTS2 of thiolase. We have now tested this prediction by affinity binding experiments, immunoprecipitation and the two-hybrid method. In addition, we have also tested the possibility that the first 55 amino acids of Peb1p might be its PTS, in view of the fact this is the only domain of Peb1p that is not part of a WD repeat.

Materials and Methods

Media and Culture Conditions

Yeasts were precultured twice in rich glucose medium (YPD) to mid log phase and then grown for 18 h in a rich medium containing glycerol and oleic acid (YPGO) as described (51) to induce peroxisomes. Glycerol supports the carbon requirement of the yeast, regardless of whether the peroxisomes are functional, and oleic acid causes peroxisome induction. When necessary to maintain plasmids, cells were precultured in minimal medium containing glucose plus any required amino acids and/or uracil (39). To test the ability of yeast to use oleic acid as the carbon source, cells were precultured in minimal medium and, at mid-log growth inoculated into liquid YNO medium (50). Cell titers were determined thereafter with a hemocytometer.

Plasmids and Yeast Strains

The plasmids used in these studies are described in Table I. All DNA synthesized by means of the polymerase chain reaction was sequenced for verification. The yeast strains used in this study are listed in Table II. The knockouts of the thiolase gene (*POT1* [20], which is identical to *FOX3* [10]) in strains JW69 and JW142 were verified by immunoblotting with anti-thiolase for the absence of thiolase protein. JW142a was isolated from a tetrad dissection of the diploid (JW142 × W303b) in order to obtain a *leu2* marker in the *pot1-peb1* double knockout strain.

Immunoprecipitation

Yeast cell extracts were prepared by vortexing with glass beads in breaking buffer (100 mM Tris-HCl, pH 7.0, 1 mM EDTA, 5% glycerol, 0.1% Triton X-100) containing protease inhibitors (45). For immunoprecipitation, 1 μg of monoclonal antibody 12CA5 against the hemagglutinin epitope (HA) was added to 50 μg of cell extract protein and incubated for 1 h at room temperature followed by 1 h at 0°C with gentle rotation. Protein A–Sepharose Cl-4B beads (20 μl of a 50% suspension Sigma, P-3391) were added and incubated for 1 h at 4°C. The beads were collected by centrifugation and washed twice with 1 ml washing buffer (100 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5% glycerol, 1% Triton X-100, 200 mM NaCl, 0.5% bovine serum albumin) and twice with breaking buffer. Bound proteins were solubilized with SDS sample buffer. Control samples received 25 μg of HA peptide before immunoprecipitation. According to the manufacturer, protein A is detached by boiling in SDS, and may bind antibodies in subsequent immunoblotting.

Two Hybrid Assay

The two-hybrid assay was based on the method of Fields and Song (12). The tested genes were fused to the DNA-binding domain (DB) of *GAL4* or to the trans-activation domain (TA) of *GAL4* (Table I) and transformed into the yeast reporter strain SFY526 (1). The transformants were grown in minimal complete glucose medium (39) to mid-log phase and homogenized with glass beads. β-Galactosidase production was measured by immunoblotting.

Affinity Binding Assay

Two oligopeptides were synthesized by the Protein Core Facility (Mount Sinai School of Medicine, New York). The wild type peptide, MSQRLQSIKDHLVLSAC, corresponds to the amino-terminal 16 residues of thiolase (16) followed by a cysteine on the COOH-terminus. The mutant peptide, MSQR**R**QSIKDHLVLSAC, is identical except for the Arg at residue five. These oligopeptides were separately coupled to TNB-agarose (Pierce, Rockford, IL) by a disulfide bond according to the manufacturer's instructions. In each case, excess peptide (1 mg) was added to 100 μl of TNB-agarose (50% suspension) bearing 0.25 μmol of TNB. Coupling was >95% efficient in both cases as judged by the release of TNB, which was measured spectrophotometrically at 410 nm. The oligopeptides, coupled to 10 μl of the beads, were incubated with 150 μg of yeast extract protein for 1 h at room temperature in buffer (100 mM sodium phosphate buffer, pH 6.8, 1 mM EDTA) with gentle rotation. The beads were then washed four times with this buffer. The peptides, together with proteins bound to them, were detached from the beads with 10 mM dithiothreitol in the same buffer.

Other Methods

The following methods were from the established protocols: immunofluorescence (49), digitonin permeabilization of cells (51), cell fractionation by differential and density gradient centrifugation (27), yeast genetics (39), yeast transformation (38), DNA manipulation (37), the catalase assay (2), and the fumarase assay (3). Immunoblotting and quantitative analysis of antigens were as described (51); secondary antibodies coupled to horse radish peroxidase for the enhanced chemiluminescence detection step

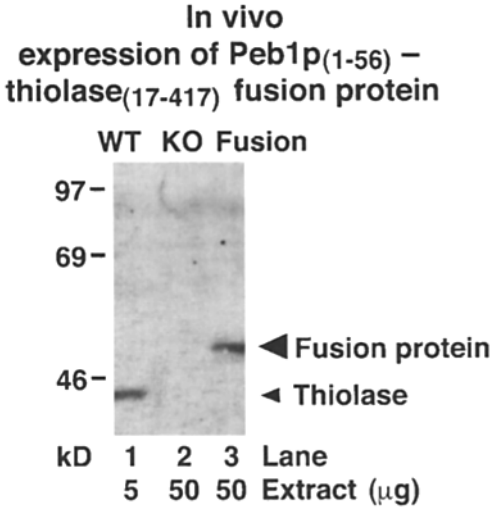

Figure 1. Expression of Peb1p$_{(1-56)}$-thiolase$_{(17-417)}$ fusion protein. Yeast cell extracts from wild type JW68-3A (*WT*), the thiolase knockout strain JW69 (*KO*), and JW69 transformed with the plasmid encoding Peb1p$_{(1-56)}$-thiolase$_{(17-417)}$ (*Fusion*, strain JW146) were separated by SDS-PAGE and immunoblotted with anti-thiolase.

were from Amersham (Arlington Heights, IL): donkey antibodies against rabbit immunoglobulins or sheep antibodies against mouse immunoglobulins were used as appropriate.

Materials

Nycodenz was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY). Digitonin (lot No. 113H0889) was from Sigma (D1407; Sigma Chemical Co., St. Louis, MO). The mouse monoclonal antibody 12CA5, the rabbit anti–thiolase, rabbit anti–phosphoglycerate kinase, and goat anti-catalase A were generous gifts from Drs. Michael Shia (Boston University School of Medicine, Boston, MA), Dr. Wolf Kunau (Bochum, Germany), Dr. Jeremy Thorner (University of California at Berkeley, Berkeley, CA) and Dr. Andreas Hartig (University of Wien, Wien, Austria), respectively. The monoclonal antibody against β-galactosidase was from Promega (Madison, WI). Other reagents were from Promega, Sigma Chemical Co., or New England Biolabs (Beverly, MA). The HA oligopeptide was synthesized by Biosynthesis, Inc. (Lewisville, TX).

Results

The experiments concerning the topogenic information that directs Peb1p to peroxisomes are described first. Thereafter, we report experiments on the interaction of Peb1p with thiolase.

The First 56 Amino Acids of Peb1p Contain a Peroxisomal Targeting Sequence

Suspecting (for reasons described in the Introduction) that the NH_2 terminus of Peb1p might contain the peroxisomal targeting signal (PTS), we fused the first 56 amino acids (Peb1p$_{(1-56)}$) in front of a passenger protein that consisted of thiolase without its first 16 amino acids (thiolase$_{(17-417)}$). Truncated thiolase was chosen because it lacks a PTS (there is no evidence for any other topogenic information in this molecule; 9, 15, 16), is a stable cytosolic protein, and is capable of entering peroxisomes when provided with an amino-terminal topogenic sequence (9, 16). The fusion protein was expressed on an episomal plasmid under the control of the *PEB1* promoter in a yeast strain from which the thiolase gene had been knocked out (Tables I and II). The fusion protein (Peb1p$_{(1-56)}$-thiolase$_{(17-417)}$) had an apparent mass of about 50 kD (Fig. 1, lane *3*), consistent with the calculated mass of 49,499 D. As expected, native thiolase was detected only in wild type cells (lane *1*). Under these expression conditions, the abundance of the fusion protein in strain JW146 was approximately 1/10 of the abundance of thiolase in wild type cells (assuming that anti-thiolase recognizes the two proteins equally well; compare loads in Fig. 1).

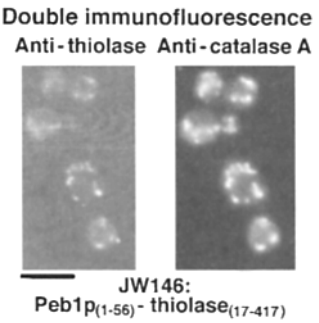

Figure 2. Intracellular location of Peb1p$_{(1-56)}$-thiolase$_{(17-417)}$ fusion protein by double immunofluorescence. Spheroplasts were first incubated with a mixture of rabbit antiserum against thiolase and goat antiserum against catalase A. After washing, they were incubated consecutively with FITC-conjugated donkey anti–rabbit immunoglobulin and TRITC-conjugated rabbit anti–goat immunoglobulin. (*Left*) observation with a green filter to show staining with anti-thiolase. (*Right*) the same cells observed with a red filter to show the staining with anti-catalase A. The specificity of the secondary antibodies was confirmed with controls in which the primary antibodies were used separately (not illustrated). Bar, 10 μm.

Immunofluorescence analysis with anti-thiolase of cells containing the fusion protein demonstrated a weak punctate staining with a pattern suggestive of peroxisomes (Fig. 2, *left*). The punctate staining observed with anti-thiolase mostly co-localized with that seen with anti-catalase A (Fig. 2, *right*), which identifies peroxisomes.

The intracellular location of the (Peb1p$_{(1-56)}$-thiolase$_{(17-417)}$) fusion protein was also investigated by subcellular fractionation. A postnuclear supernatant fraction was prepared from strain JW146 and separated into an organelle pellet (mainly mitochondria and peroxisomes, ML) and a supernatant (containing cytosol, PS). The fusion protein was mainly (90%) located in the organelle pellet (Fig. 3 *A*).

This organelle fraction was subjected to equilibrum density Nycodenz gradient centrifugation to separate peroxisomes from mitochondria. As shown in Fig. 3 *B* (*top*), the peroxisomes (as marked by catalase activity) were well separated from mitochondria (as marked with cytochrome *c* oxidase). The fusion protein was found at the bottom of the gradient together with catalase (Fig. 3 *B*, *bottom*). The fusion protein was enriched, relative to catalase, on the denser side of the peroxisome peak, for reasons as yet unknown. It was also noticed that a band below the fusion was present in the mitochondrial fraction (fractions 4–6); the origin of this band is unknown. In a second experiment, in which the peroxisomes sedimented only half way down the gradient, the fusion protein again accompanied the peroxisomes (data not shown). These cell fractionation data confirm the immunofluorescence double-labeling data that the fusion protein is localized in peroxisomes.

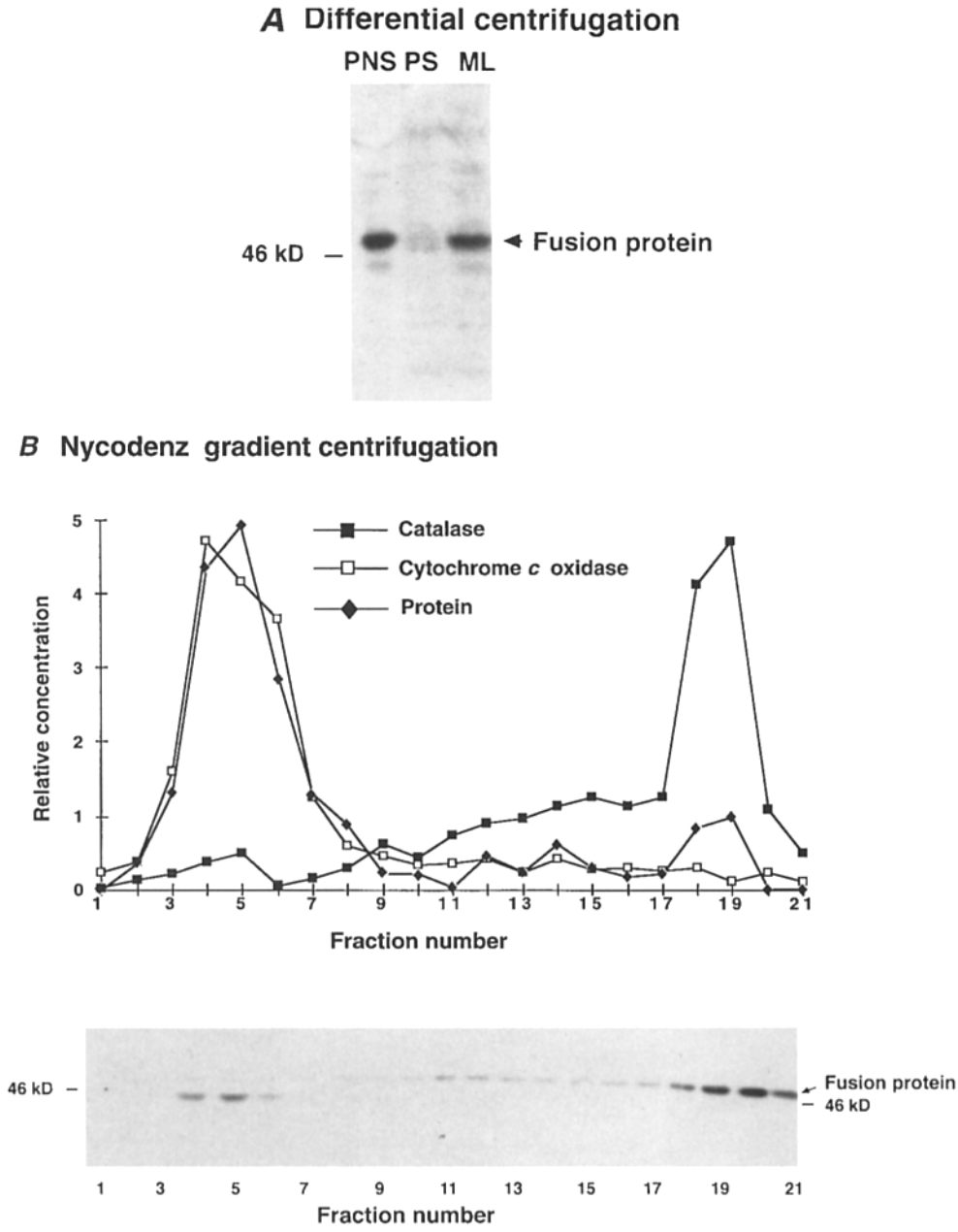

Figure 3. Localization of the Peb1p$_{(1\text{-}56)}$-thiolase$_{(17\text{-}417)}$ fusion protein by subcellular fractionation of JW146. (*A*) Differential centrifugation. Equal percentages of postnuclear supernatant (*PNS*), 25,000 *g* supernatant (*PS*), and 25,000 *g* pellet (*ML*) were analyzed by SDS-PAGE, followed by immunoblotting with anti-thiolase to detect the fusion protein. The ML sample contained 3.1 μg of protein. Recovery = 87%. (*B*) Nycodenz gradient centrifugation of the 25,000 *g* organelle pellet. (*Top*) Distribution of catalase activity, cytochrome *c* oxidase activity and protein in the gradient. The ordinate represents relative concentrations as calculated according to de Duve (7). 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. (*Bottom*) Distribution of the fusion protein in the fractions, detected by immunoblotting. 30 μl of each fraction was analyzed (25 μg of protein in the case of fraction 5). Recovery = 68%.

The First 56 Amino Acids of Peb1p Are Required for the Peroxisomal Targeting of Peb1p

We have previously observed that Peb1p, tagged at its carboxy terminus with three copies of the hemagglutinin epitope tag, and expressed at the wild type level under the control of its own promoter (strain JW86), is found entirely within peroxisomes (within experimental error) (50). We now constructed a gene encoding a truncated version of this tagged protein, lacking amino acids 6–55, by means of the polymerase chain reaction. It was similarly expressed under the control of its own promoter, in the *peb1* knockout strain JW75, on an integrating plasmid (strain JW162). The gene product, named Peb1p$_{(\Delta 6\text{-}55)}$-HA$_3$, migrated with an apparent mass of 41 kD, consistent with its predicted mass of 40,773 D (Fig. 4, lane *2*). The expression level of Peb1p$_{(\Delta 6\text{-}55)}$-HA$_3$ was comparable to that of the full-length protein in JW86 (Fig. 4, lane *1*).

The intracellular location of Peb1p$_{(\Delta 6\text{-}55)}$-HA$_3$ was analyzed by the progressive permeabilization of spheroplasts with increasing concentrations of digitonin (Fig. 5). As we have shown previously (51), very low concentrations of digitonin are sufficient to release the cytosolic enzyme, phosphoglycerate kinase, from the spheroplasts because of the abundance of sterols in plasma membrane. Much higher concentrations of digitonin are required to permeabilize the peroxisomal membrane (as indicated by the release of catalase activity to the supernatant) and still more digitonin is needed to permeabilize mitochondria (as marked by fumarase activity). The release pattern of Peb1p$_{(\Delta 6\text{-}55)}$-HA$_3$ was very similar to that of phosphoglycerate kinase (Fig. 5 *B*), indicating the cytosolic location of

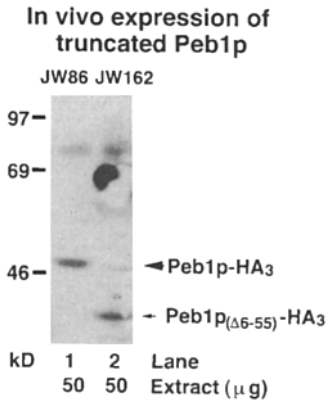

Figure 4. In vivo expression of full length and truncated Peb1p-HA_3. Immunoblot detection with monoclonal antibody 12CA5. Lane *1*, full-length Peb1p-HA_3 in strain JW86. Lane *2*, the truncated version, lacking residues 6-55 (Peb1p$_{(\Delta 6\text{-}55)}$-HA_3), in strain JW162.

this truncated protein. This contrasts with full-length Peb1p-HA_3 which is inside peroxisomes and emerges with catalase (Fig. 5 *A*). These data indicate that the NH_2-terminal amino acid residues 6–55 are essential for the import of Peb1p into peroxisomes.

Truncated, Cytosolic Peb1p Is Nonfunctional for Importing Thiolase into Peroxisomes

Digitonin permeabilization (Fig. 5 *B*) together with immunofluorescence analyses (Fig. 6 *A*) of cells expressing the truncated Peb1p (strain JW162) indicated that thiolase was in the cytosol. This contrasts with the situation in cells expressing the full-length tagged protein, which have been shown to partially package thiolase into peroxisomes (Figs. 5 *A* and 6 *A*, strain JW86, discussed in reference 50).

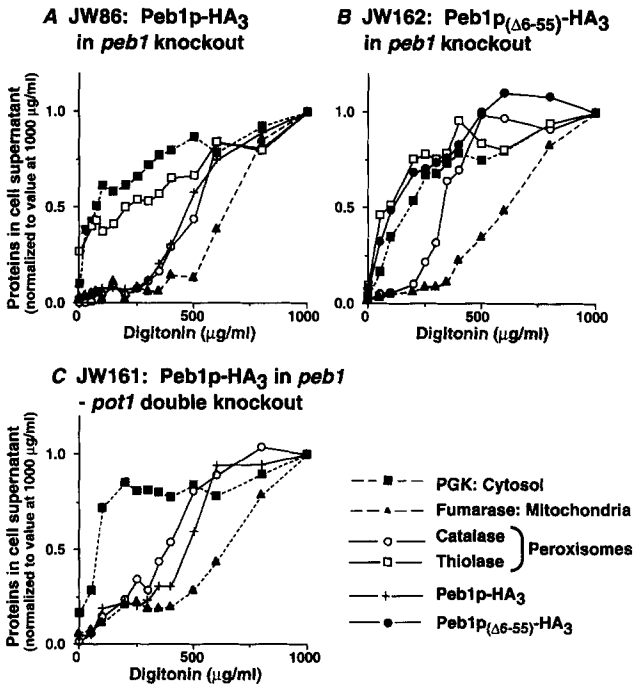

Figure 5. Subcellular localization of intracellular proteins by digitonin titration. Aliquots of spheroplasts were incubated with the indicated concentrations of digitonin for 20 min at 4°C and then centrifuged to pellet the cells. The supernatants were analyzed for released proteins. The activity of the peroxisomal enzyme, catalase, and the mitochondrial enzyme, fumarase, were assayed enzymatically. The cytosolic marker, phosphoglycerate kinase (*PGK*), as well as thiolase and 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.

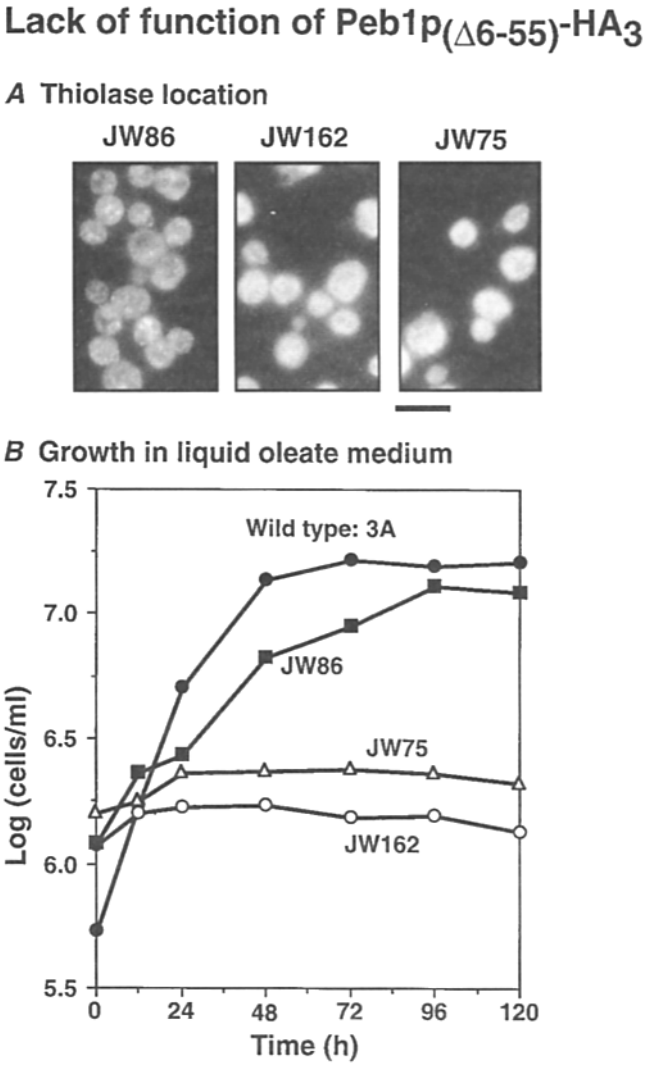

Figure 6. Tests of Peb1p$_{(\Delta 6\text{-}55)}$-HA_3 function. (*A*) Intracellular location of thiolase determined by immunofluorescence. Spheroplasts were incubated with rabbit anti–thiolase followed by FITC-conjugated donkey anti–rabbit immunoglobulins. Bar, 10 μm. (*B*) Ability of cells to grow in medium with oleic acid as the carbon source (YNO medium). JW75, the *peb1* null mutant. JW86, the null mutant transformed with YipPEB1-HA_3, a single copy integrating plasmid containing full-length *PEB1* with its normal control elements. JW162, the null mutant transformed with YcpPEB1Δ-HA_3, a single copy centromere plasmid containing truncated *PEB1* with its normal control elements.

Further evidence for the lack of function of truncated Peb1p came from a test of its capacity to allow cells to grow on oleate as the sole carbon source. Wild type yeast can grow on oleate (YNO plates) by means of the peroxisomal β-oxidation of this fatty acid. The *peb1* knockout strain lacks this ability, presumably because thiolase is missing from peroxisomes (Fig. 6 *B*, strain *JW75*) (50). Full-length epitope-tagged Peb1p partially restores this

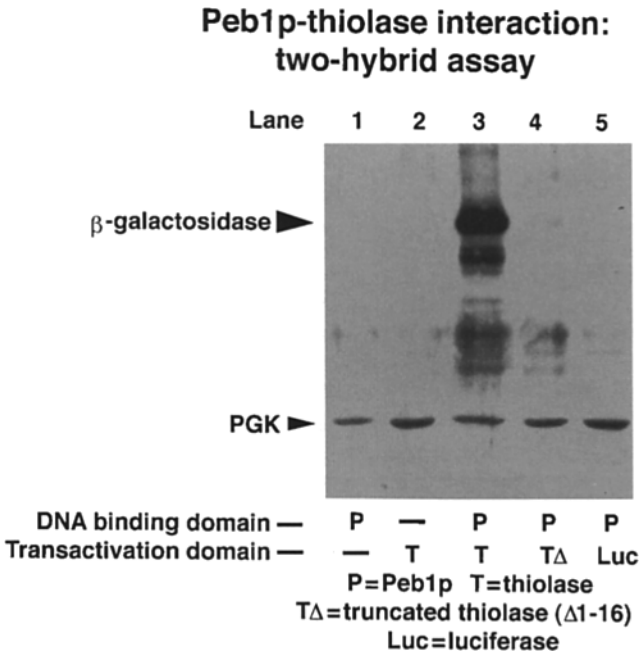

Figure 7. Peb1p–thiolase interaction detected by the two-hybrid assay. The DNA binding domain (DB) of Gal4p was fused to Peb1p (*P*) or nothing (−) as indicated. The transactivation (TA) domain of Gal4p was fused to nothing (−), thiolase (*T*), truncated thiolase lacking the first 16–amino acid residues (*TΔ*) or luciferase (*Luc*). These GAL4 fusion proteins were expressed either alone, or in combination as indicated at the bottom of the figure. Protein–protein interaction, causing Gal4p-dependent expression of β-galactosidase, was detected with anti-β-galactosidase antibody. The amount of PGK, also detected by immunoblotting, served as a loading control among the lanes.

ability (strain *JW86*; see reference 50 for more information) but truncated Peb1p did not (Fig. 6 *B*, strain *JW162*).

Peb1p Is Found in Peroxisomes Whether Thiolase Is Synthesized or Not

Full-length, epitope-tagged Peb1p was expressed at wild type levels in a double knockout strain of *peb1* and the thiolase gene, *pot1*. Digitonin cell fractionation was carried out to determine the subcellular location of the Peb1p-HA_3. Its release at different concentrations of digitonin was comparable to that of catalase (Fig. 5 *C*, strain *JW161*), indicating that the Peb1p was located in the peroxisomes in the thiolase-deficient cell. Therefore, the peroxisomal location of Peb1p does not require that thiolase is being synthesized and imported into peroxisomes.

Peb1p Is a Thiolase PTS2 Receptor

The hypothesis that Peb1p is a receptor for the PTS2 targeting sequence of thiolase was tested by three different methods: the yeast two-hybrid assay, affinity chromatography, and co-immunoprecipitation.

Peb1p Interacts with Thiolase

The possible interaction of Peb1p and thiolase was tested by means of the two hybrid assay (12). This assay uses a yeast strain in which the bacterial LacZ gene was integrated into a chromosome and requires the function of the GAL4 protein for its expression. When the DNA-binding domain (DB) and the transactivation domain (TA) of Gal4p are co-expressed from separate plasmids, Gal4p is nonfunctional and no β-galactosidase is synthesized. The DNA binding domain of Gal4p was fused to Peb1p and the transactivation domain was fused to thiolase. Coexpression of these two fusion proteins caused a massive production of β-galactosidase (Fig. 7, lane *3*). Single expression of either fusion protein (together with the unfused other domain of Gal4p) did not induce β-galactosidase (lanes *1* and *2*). As an additional control, the transactivation domain of GAL4 was fused to the luciferase gene and coexpressed with the GAL4 (DB)-PEB1 fusion gene: no expression of β-galactosidase was detected (lane *5*). These results indicate that Peb1p interacts with thiolase, which has a PTS2, but not with luciferase which has a carboxyl-terminal PTS1.

Peb1p's Interaction with Thiolase Requires the First 16–amino Acid Residues of Thiolase

The transactivation domain of Gal4p was fused to a truncated version of thiolase lacking the first 16 amino acids. This fusion was co-expressed with the previously tested DNA-binding domain of Gal4p attached to Peb1p: no induction of β-galactosidase occurred (Fig. 7, lane *4*). These data indicate that the PTS2 of thiolase is essential to the Peb1p–thiolase interaction.

The First 16 Amino Acids of Thiolase are Sufficient for Peb1p Binding

An oligopeptide consisting of the first 16 amino acids of thiolase followed by a cysteine was synthesized and coupled covalently at its COOH-terminus to agarose beads (via an S-S linkage, see Materials and Methods). These beads were used for affinity chromatography with an extract of yeast strain JW88, which contains large amounts of epitope-tagged Peb1p. A substantial amount of Peb1p bound to these beads and (after extensive washing) was detached (together with the peptide) by dithiothreitol (Fig. 8 *A*, lane *2*). The vast bulk of the proteins in the yeast extract did not bind (Fig. 8 *B*, lanes *1* and *3*). Comparison with the starting material (Fig. 8 *A*, lane *1*) by densitometry indicates that about 20% of the Peb1p in the cell extract was recovered in highly purified form by this affinity binding procedure.

As a control, a mutant oligopeptide was synthesized and coupled to agarose beads. The mutant peptide was identical to the wild type peptide except that the fifth amino acid was changed from leucine to arginine. It has been demonstrated that this amino acid substitution (L5R) reduces the import of thiolase into peroxisomes in vivo to 9–12% (16). As seen in Fig. 8 *A*, lane *3*, much less amount of Peb1p bound to the L5R peptide. Densitometry indicated that it amounted to 3% of the applied Peb1p. This result demonstrates that an amino acid among the first 16 that is essential for efficient thiolase topogenesis is also required for efficient Peb1p binding.

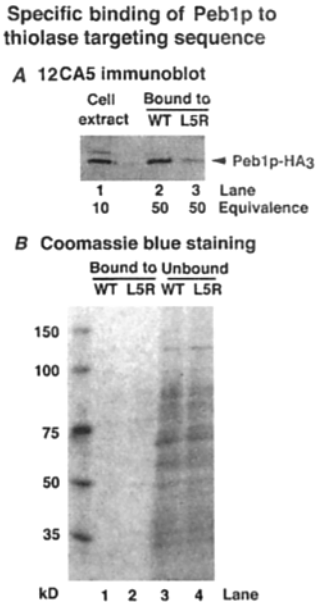

Figure 8. Specific binding of Peb1p to the thiolase targeting sequence. A chemically synthesized peptide, corresponding to the first 16 amino acids of thiolase, followed by cysteine (*WT*) was coupled by a disulfide linkage to agarose beads as described in Materials and Methods. A mutant peptide, differing only in the replacement of leucine at residue 5 by arginine (*L5R*), was similarly coupled. Yeast cell extract (150 μg) from strain JW88 expressing epitope-tagged Peb1p, was applied to each type of beads. After binding for 60 min, removal of the unbound proteins and repeated washing, the peptides and proteins bound to them were detached from the beads with dithiothreitol. (*A*) One third of the proteins bound to wild type peptide (*WT*) or mutant peptide (*L5R*) were analyzed by immunoblotting with antibody 12CA5. For comparison, 10 μg of the cell extract applied to the beads was analyzed in lane *1*. (*B*) One third of the total proteins bound to the peptides (lanes *1* and *2*) or not bound (lanes *3* and *4*) were analyzed by SDS-PAGE and Coomassie blue staining.

Co-immunoprecipitation of a Peb1p–Thiolase Complex

A co-immunoprecipitation experiment was designed with the following concern in mind. Peb1p, as a component of the thiolase import machinery, would be expected to interact only transiently with its substrate. In a normal, import-competent cell, a Peb1p–thiolase complex might represent only a very small part of the total Peb1p protein, and therefore might be hard to detect. However, in a cell lacking peroxisomes, where the import process is defective, this complex might have a longer half-life. In this case, the complex would be more abundant and easier to detect.

Peb1p-HA_3 was over-expressed in *peb4-1*, a mutant which contains peroxisome membrane ghosts (35, 49), but all peroxisomal matrix proteins tested are found in the cytosol (49, 51). Peb1p, in this new strain JW89, was also found in the cytosol (data not shown). Peb1p was efficiently immunoprecipitated from a cell extract with monoclonal antibody 12CA5 (Fig. 9 *B*, compare lanes *3*, *6*, and *8*). A significant amount of thiolase was also found in this immunoprecipitate (Fig. 9 *A*, lane *3*). As a control against nonspecific binding, excess HA peptide was added to a duplicate aliquot of the extract prior to immunoprecipitation. The excess HA prevented the immunoprecipitation of Peb1p-HA_3 (Fig. 9 *B*, lane *4*) and also of thiolase (Fig. 9 *A*, lane *4*). These data demonstrate that thiolase was recovered in the immunoprecipitate by virtue of its association with the epitope-tagged Peb1p. An extract from a similar strain (JW88), likewise overproducing tagged Peb1p, but in which peroxisome biogenesis proceeds normally, was also used for immunoprecipitation (Fig. 9). Peb1p was efficiently immunoprecipitated from this extract (Fig. 9 *B*, lane *1*), but was not accompanied by thiolase (*A*, lane *1*). These data are consistent with the hypothesis that, in import competent cells, the Peb1p–thiolase complex is too transient to be detected.

Discussion

Peb1p-PTS2 Binding

These experiments have clearly demonstrated an interaction between Peb1p and thiolase. The yeast two-hybrid assay and co-immunoprecipitation showed that Peb1p binds to thiolase and that this binding requires the first 16 amino acids of thiolase, which constitute the type 2 targeting sequence (PTS2). Peb1p did not bind to luciferase, which is targeted by a PTS1. The PTS2 is sufficient for Peb1p binding, as shown by the highly-specific affinity binding of Peb1p to an oligopeptide, consisting of the first 16 amino acids of thiolase, coupled to agarose beads. This binding correlated with the topogenic function of the peptide: a single amino acid change (leucine 5 to arginine), which is

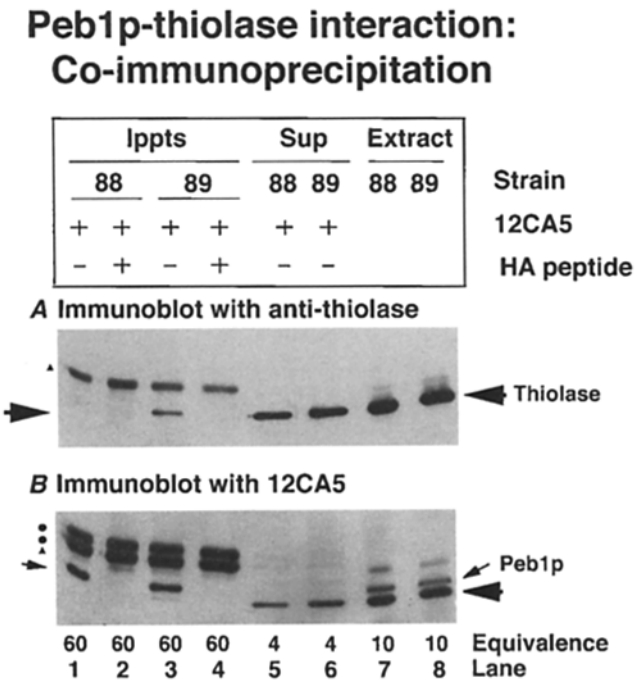

Figure 9. Co-immunoprecipitation of thiolase with Peb1p. Peb1p-HA_3 was immunoprecipitated (Ippts) from 60 μg of cell extract of strain JW88 (lanes *1* and *2*) or strain JW89 (lanes *3* and *4*) with 1 μg of monoclonal antibody 12CA5 and analyzed by SDS-PAGE. For comparison, one fifteenth of the supernatants (*Sup*) after the immunoprecipitation (lanes *5* and *6*) and one sixth (10 μg) of the starting cell extracts (*Extract*) were also analyzed. As a control, an excess of HA peptide was added before the immunoprecipitation to some duplicates (lanes *2* and *4*). The immunoprecipitates were subjected to SDS-PAGE and transblotted to nitrocellulose. The nitrocelluse membrane was first probed with rabbit anti–thiolase (*A*) and, subsequently (without stripping of the nitrocellulose), with 12CA5 antibody (*B*). Thiolase is denoted with thick arrows. Tagged Peb1p is denoted with fine arrows. Binding of rabbit anti-thiolase to Protein A (detached from the Sepharose by the SDS) is visible in *A* (*triangle*). As expected, the 12CA5 antibody present in the immunoprecipitates, appears in *B* (*dots*).

known to reduce thiolase import in vivo 8- to 11-fold (16), also reduced Peb1p binding about 7-fold. Taken together, these data demonstrate that the PTS2 of thiolase (its first 16 amino acids) is necessary and sufficient to interact with Peb1p.

The PTS of Peb1p

Peb1p has been demonstrated previously to be an intraperoxisomal protein (50). We have now found that Peb1p contains a PTS within its first 56 amino acids. This NH_2-terminal domain (which is the only part of Peb1p not in a WD repeat) is necessary for Peb1p to reach peroxisomes, and sufficient to target a passenger protein (truncated thiolase) to peroxisomes. This domain shows no obvious homology to the thiolase PTS2; in particular it lacks the RL and HL dipeptides (or close approximations thereof) that are thought to be required for PTS2 topogenesis. In addition, Peb1p probably does not play a role in its own import because a *peb1* knockout strain can be restored to wild type function by the reintroduction of the *PEB1* gene on a plasmid (50). The NH_2-terminal topogenic domain of Peb1p also does not appear to show any obvious similarity to the internal domains of *Candida tropicalis* acyl-CoA oxidase (21, 42) or to the internal domain of *S. cerevisiae* catalase A (24) that target these proteins to peroxisomes. The Peb1p PTS appears to be novel.

The Role of Peb1p as a PTS2 Receptor

Where in the cell does the interaction between Peb1p and the PTS2 occur and how does Peb1p function to bring thiolase into the peroxisome? We have considered two models, a co-import model and an intraperoxisomal receptor model. One version of a co-import model was suggested by Marzioch et al. (28) on the basis of their data that Peb1p, epitope-tagged at its aminoterminus, is found in the cytosol in cells not expressing thiolase. This implied that Peb1p reached peroxisomes by virtue of its association with thiolase, perhaps taking advantage of thiolase's PTS2 to reach peroxisomes. This version of the co-import model is untenable in view of the fact that Peb1p has its own PTS and that Peb1p clearly is in peroxisomes in a thiolase knockout strain (Fig. 5).

Co-import?

We might speculate on another hypothetical version of a co-import model. It is theoretically possible that thiolase could bind to Peb1p in the cytosol via its PTS2, and then ride piggyback into peroxisomes, directed to the peroxisome by Peb1p's PTS. This would require that the cell make at least as many molecules of Peb1p as of thiolase (or at least half as many if all thiolase molecules were to enter peroxisomes as dimers; 15). The steady state abundance of Peb1p appears to be far less than that of thiolase. The polypeptide composition of peroxisomes isolated from a *peb1* knockout strain has been compared with the composition of wild type peroxisomes by silver staining: thiolase was missing, as expected, but no polypeptide with the mass of Peb1p was missing (28). This suggests that Peb1p, like many other proteins required for peroxisome biogenesis, (e.g., PAF-1; 40), is present at a very low abundance. We may estimate the abundance of Peb1p from the data of Fig. 1. The fusion protein ($Peb1p_{(1-56)}$-$thiolase_{(17-417)}$) was expressed under the control of the *PEB1* promoter on a multi-copy plasmid and its abundance, estimated by immunoblotting with anti-thiolase, was 1/10 that of thiolase itself. Since there are 20–50 copies of the plasmid per cell, the expression level of the fusion protein from a single copy of the gene would be 1/200 to 1/500 of thiolase. Let us assume that anti-thiolase recognizes the fusion protein equally well as thiolase, which is likely because the antibody recognizes truncated thiolase lacking its first 15 amino acids just as well as the full-length thiolase (10). Let us also assume that the steady state expression level of $Peb1p_{(1-56)}$-$thiolase_{(17-417)}$ is the same as the expression of Peb1p. This seems likely because the promoters are the same and the beginning of the proteins (including the translation initiation site) are the same, and peroxisomal proteins turn over synchronously, at least in rat liver (33). In this case, we may infer that Peb1p in the wild type cell is 200 to 500 times less abundant than thiolase. This suggests that Peb1p is playing a catalytic role, not a carrier role. It is theoretically possible that Peb1p might shuttle back out of peroxisomes and re-enter carrying another thiolase molecule, but there is no experimental evidence for protein export from peroxisomes.

An Intra-peroxisomal PTS2 Receptor

The simplest model that is consistent with all of the experimental data is that Peb1p functions as an intra-peroxisomal PTS2 receptor. After all, Peb1p is found in peroxisomes and it binds the PTS2. In vivo, as suggested previously (50), Peb1p might bind newly-synthesized thiolase's PTS2 as it appears at the inside face of the peroxisome membrane, and then, perhaps together with other proteins, Peb1p might "pull" thiolase inside. There is a formal similarity of this proposed mechanism with the role of mitochondrial HSP70 in the import of mitochondrial proteins (22).

If this is correct, we speculate that another PTS2 receptor might function in the cytosol or on the outer surface of the peroxisomal membrane in the initial insertion of the NH_2-terminus of thiolase through the peroxisomal membrane. Further research is required to investigate this possibility.

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