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Peroxisome biogenesis mutants and gene analysis in *Saccharomyces cerevisiae*

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Peroxisome Biogenesis Mutants and Gene Analysis in *Saccharomyces cerevisiae*

By

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The City University of New York

Abstract

Peroxisome Biogenesis Mutants and Gene Analysis in *Saccharomyces cerevisiae*

By

Jing Wei Zhang

Advisor: Professor Paul B. Lazarow

The peroxisome is a nearly ubiquitous organelle in eukaryotic cells. It has vital functions in the cell, including H_2O_2-based respiration, fatty acid ß-oxidation, plasmalogen synthesis and cholesterol metabolism. A class of human genetic diseases is caused by the deficiency of peroxisome biogenesis. The goal of this project is to use the yeast, *Saccharomyces cerevisiae,* as a model system to study peroxisome biogenesis.

I first devised a positive selection procedure that identifies mutants lacking peroxisomes or peroxisomal functions. Immunofluorescence methods for yeast were simplified so that these mutants could be efficiently analyzed for impairments in

peroxisome biogenesis. With these tools, I have identified eleven peroxisome biogenesis (*peb*) mutants which were sorted into five complementation groups.

I then developed a new gentle cell fractionation procedure which employs digitonin titration for the selective permeabilization of yeast plasma and intracellular membranes. This permits the intracellular distribution of proteins to be determined accurately, without mechanical breakage of membranes.

These five groups of *peb* mutants were analyzed with immunofluorescence, electron microscopy, immunoelectron microscopy and this new cell fractionation method. The biochemical and morphological data were consistent. Mutants from two groups lack recognizable peroxisomes. All the peroxisomal proteins examined are mislocated in the cytosol in them. Two groups contain peroxisomes, but are selectively defective in packaging newly synthesized peroxisomal proteins: *peb1* fails to package thiolase, but the import of catalase and acyl-CoA oxidase into peroxisomes is normal; *peb5* does not package catalase, but packages thiolase and acyl-CoA oxidase. These two mutants also show striking

This work is dedicated to

My father, my mother

My lovely wife Hong Wang

My daughter Michelle and My son Cliff

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AOx	acyl-CoA oxidase
bp	base pair
D	dextrose
DAB	3,3'-diaminobenzidine
EMS	ethylmethane-sulfonate
G	glycerol
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HA	The epitope from hemagglutinin of Influenza virus encoding YPYDVPDYA.
kDa	kilodalton
N	yeast nitrogen base
nsLTP	non-specific lipid transfer protein
O	oleate
ORF	open reading frame
P	bacto-peptone
pas	peroxisome assembly
peb	peroxisome biogenesis
PGK	phosphoglycerate kinase

PMSF	phenylmethylsulfonyl fluoride
RCDP	the rhizomelic type of chondrodysplasia punctata
T	Tween 40
UTR	untranslated region
Y	yeast extract

Chapter 1

Historical Review and Background

General Aspects

The peroxisome was discovered in the early 1950's in mouse kidney cells by electron microscopy (Rhodin, 1954). Termed *microbody*, it was renamed in 1966 as *peroxisome* by Christian de Duve, based on its role in H_2O_2-based respiration (de Duve and Baudhuin, 1966).

Peroxisomes are bounded by a single membrane and generally have a diameter of 0.1 to 1 µm. The enzyme content and function of peroxisomes varies among different species. There are two major functions more or less common to all peroxisomes: H_2O_2 based respiration and ß-oxidation of fatty acids (Lazarow and Moser, 1989).

H_2O_2-based respiration was the first function of peroxisomes to be discovered by de Duve and coworkers (Baudhuin et al., 1964),

and is seen in peroxisomes from every species. This pathway is based on the formation of H_2O_2 by a variety of oxidases and the decomposition of H_2O_2 by catalase. Several oxidases have been identified in peroxisomes in different species, including urate oxidase, D-amino acid oxidase, alcohol oxidase, and acyl-CoA oxidase (de Duve and Baudhuin, 1966; Roggenkamp et al., 1975, Osumi and Hashimoto, 1978).

Peroxisomal fatty acid ß-oxidation was first discovered in germinating seeds (Cooper and Beevers, 1969). It was found that it also exists in animal cells by Lazarow and de Duve in 1976 (Lazarow and de Duve, 1976). Since then, peroxisomal ß-oxidation of fatty acids has been found in a wide variety of species. In plants and microorganisms, fatty acid ß-oxidation only occurs in peroxisomes. In animal cells, both mitochondrial and peroxisomal fatty acid ß-oxidation are present, but the enzymes are encoded by different genes in these two systems. The pathway of peroxisomal fatty acid ß-oxidation is shown in Figure 1-1. Five enzymes are involved in this pathway, including acyl-CoA synthetase, acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and

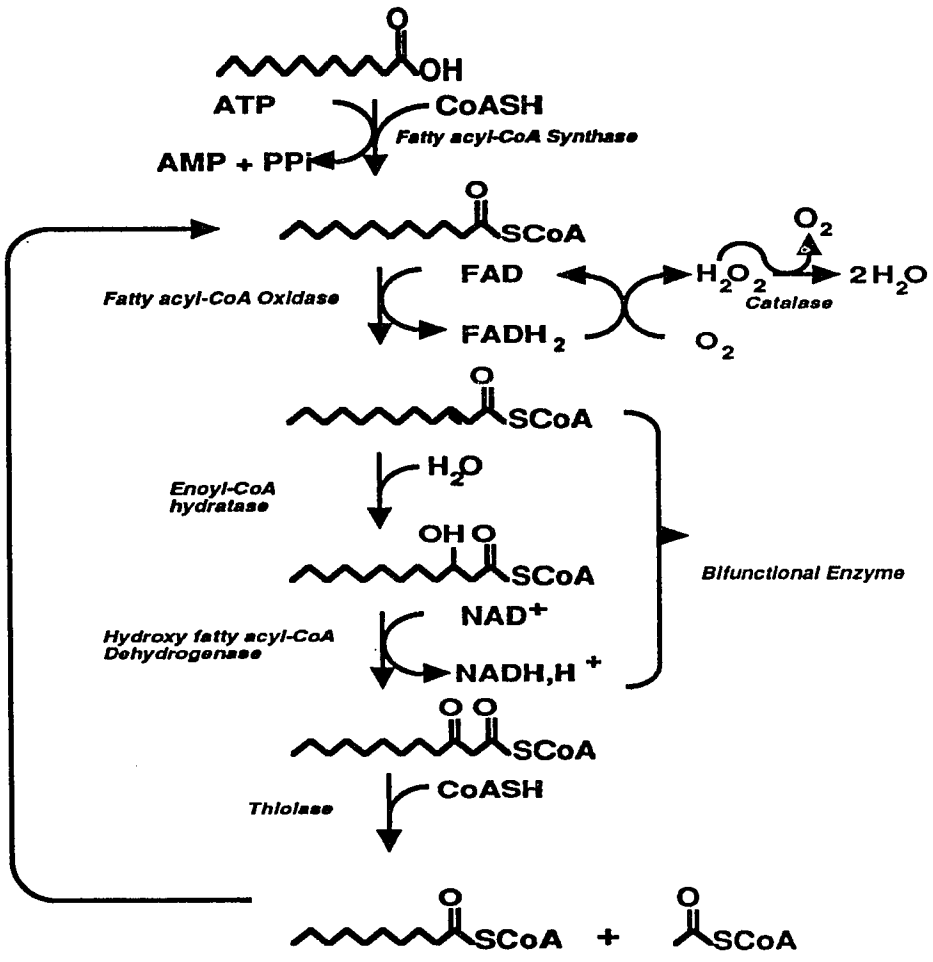


Figure 1-1. Peroxisomal fatty acid b-oxidation pathway.

thiolase. All of them are located in peroxisomes. During each cycle of oxidation, one molecule of H_2O_2 is generated, which is degraded by catalase. In plants and microorganisms, the product of fatty acid ß-oxidation, acetyl-CoA, is condensed to succinate via the glyoxylate cycle. The succinate is further converted to glucose through the gluconeogenic pathway (elsewhere in the cell). In animal cells, the acetyl-CoA generated by peroxisomal β-oxidation is also believed to be available for anabolic purposes. As peroxisomal ß-oxidation in animal cells can only oxidize fatty acids longer than six carbons, and the very long chain fatty acid is the preferred substrate for this pathway (Osmundsen et al., 1979), it is thought by some that this pathway is mainly for chain shortening of very long chain fatty acids (Osmundsen et al., 1980).

Two other important functions of peroxisomes in animal cells are ether lipid (plasmalogen) synthesis, and cholesterol metabolism (Lazarow and Moser, 1989). Ether lipids, mainly plasmalogen, are a component of membrane phospholipids, and are enriched in nerve cells (Wykle, 1977). The first two enzymes of ether lipid synthesis, dihydroxyacetone phosphate acyltransferase (DHAP-AT) and alkyl

DHAP synthetase are located in peroxisomes (Hajra and Bishop, 1982).

Peroxisomes also have been shown to be involved in cholesterol metabolism. Biochemical and cell fractionation studies indicate that at least two enzymes involved in cholesterol synthesis, hydroxymethylglutaryl-CoA (HMG-CoA) reductase (Keller et al., 1986) and mevalonate kinase (Stamellos et al., 1992), are located in peroxisomes. The cell lines from Zellweger patients have decreased synthesis of cholesterol (Hodge et al., 1991), suggesting that normal peroxisomes are required for the cholesterol synthesis. Peroxisomes also play an essential role in the catabolism of cholesterol, the formation of bile acids. The conversion of 3α, 7α, 12α-trihydroxycholic acid (THCA) to bile acid is a peroxisomal process, involving the oxidative cleavage of the side chain through the ß-oxidation pathway (Prydz et al., 1986). Patients with Zellweger syndrome accumulate THCA in their blood due to the deficiency of this function (Lazarow and Moser, 1989).

In liver cells of rodents, a lot of structurally dissimilar xenobiotics can induce the proliferation of peroxisomes. They are

therefore called peroxisome proliferators. The induction by peroxisome proliferators is species-specific. Monkeys and humans are not sensitive to these proliferators (Rodricks et al., 1987, Blaauboer et al., 1990). The peroxisome proliferator activated receptors (PPAR) have been isolated from different species (Keller and Wahli, 1993; Issemann and Green, 1990, Andre and Small, 1993). A peroxisome proliferator binding protein (Lalwani et al., 1987), which turns out to be identical with a member of the 70 kD heat shock protein family (Alvares et al., 1990) has also been isolated. The PPAR and peroxisome proliferator binding protein are probably involved in the induction of peroxisomes.

Peroxisome Biogenesis

Peroxisomes do not contain their own genome. All peroxisomal proteins are encoded by nuclear genes. Historically, peroxisomes were thought to form by budding from the endoplasmic reticulum. Subsequent studies using biochemical and cell biology techniques indicated that the peroxisome is formed by division, not through ER budding. All peroxisomal proteins are synthesized on free ribosomes, most of them at their final size, and are translocated to

preexisting peroxisomes (see review, (Borst, 1989; Lazarow and Fujiki, 1985; Small, 1993)). Whether the peroxisome can ever be formed de novo still remains to be tested.

Targeting information: Like the proteins destined for the ER and mitochondria, proteins intended for peroxisomes must contain a targeting signal that dictates its destination. Recent studies indicate that peroxisome proteins use at least three kinds of topogenic sequences for their targeting, which are C-terminal SKL tripeptide, N-terminal peptide and internal sequence.

The SKL-like sequence in the extreme carboxyl-terminal is the best characterized. In 1987, Subramani and coworkers (Keller et al., 1987) first found that when animal cells were transfected with luciferase, a peroxisomal enzyme existing in the lantern organ of fireflies, the protein appeared in the peroxisomes. Subsequent studies showed that the targeting signal for directing luciferase to the peroxisome resides in the C-terminal tripeptide, serine-lysine-leucine (SKL). This sequence is both required and sufficient to direct a cytosolic protein to the peroxisome. The SKL only functions when residing on the extreme C-terminal of the protein. Site-

directed mutagenesis established the consensus sequence as (Ser/Ala /Cys)-(Lys/Arg/His)-Leu (Gould et al., 1987; Gould et al., 1989). A lot of peroxisomal proteins from different species of eukaryotic cells have been found to contain this tripeptide or its variant (Gould et al., 1989). Antibody made against the SKL tripeptide recognizes peroxisomes in several species of eukaryotic cells (Keller et al., 1991).

While most peroxisomal proteins are synthesized in their full size on ribosomes, three peroxisomal proteins, including mammalian peroxisomal thiolase (Osumi et al., 1991; Swinkels et al., 1991), mammalian non-specific lipid transfer protein (nsLTP) (Tsuneoka et al., 1988), and malate dehydrogenase from watermelon glyoxysomes (the peroxisome equivalent in plants) (Gietl, 1990), contain an N-terminal cleavable sequence. Sequence comparison of these N-terminal peptides showed substantial homology (Osumi et al., 1991). Gene fusion experiments have shown that the N-terminal sequence in rat peroxisomal thiolase is required and sufficient to target a cytosolic protein to peroxisomes (Osumi et al., 1991; Swinkels et al., 1991). Therefore, the N-terminal cleavable sequence in rat

peroxisomal thiolase is the targeting sequence. The other two proteins probably also use the N-terminal peptide for their targeting.

There are a lot of peroxisomal proteins which have neither of the two topogenic sequences discussed above. For most of them, the targeting signal remains to be determined. Using an *in vitro* import system, Small *et al.* (1988) have defined two internal sequences in acyl-CoA oxidase from *Candida tropicalis* which can target the cytosolic protein to peroxisomes *in vitro*. This conclusion was supported by subsequent in vivo experiments (Kamiryo et al., 1989).

The fact that peroxisomal proteins use more than one targeting signal suggests that there is probably more than one pathway or branches of a pathway, for the import of peroxisomal proteins.

Import machinery: Great efforts have been taken to understand the mechanism of peroxisomal protein import recently. Two kinds of methodology have been employed: in vitro studies and genetic approaches.

Lazarow and coworkers (Imanaka et al., 1986; Small and Lazarow, 1987) established the first in vitro peroxisome import assay by using isolated peroxisomes and in vitro translated proteins. Import is judged by protease protection. Mitochondria are used as a control organelle for the specificity. Using this system, they found that protein import into peroxisomes is a two-step process: association with peroxisomes (probably via receptor(s)), and translocation. The first step can occur at 0° C and is ATP-independent. Translocation occurs at 25° C or above and is ATP-dependent. These results suggest that at least two components are required for import: receptor(s) and translocator(s).

Very recently, Wendland and Subramani (1993) developed a streptolysin O-permeablized cell system retaining the peroxisomal protein import competence in CHO cells. Luciferase and SKL-conjugated albumin were used to monitor the import into the organelle. Using this system, they found that the peroxisomal protein import is ATP dependent, temperature dependent, and cytosol dependent. It was also found that the cytosol contains SKL binding sites and is heat sensitive, indicating protein(s), rather than small

organic molecules, is the effector in it.

Studies of the requirement of membrane potential for peroxisomal protein import have given variable results. Bellion and Goodman (1987) have shown that proton ionophores can prevent the assembly and import of AOx into peroxisomes in *Candida boidinii*. More recently, that same group has shown that this disruptive effect on the import of AOx can not be accounted by the lowering of the ATP concentration of yeast cells caused by the ionophores (Sohaskey et al., 1993). These results suggest that a peroxisomal proton-motive force is responsible for the import of proteins into peroxisomes in this yeast. However, the in vitro import studies using purified rat peroxisomes (Imanaka et al., 1987) indicate that the import of proteins into peroxisomes is not membrane-potential dependent. Whether these two different conclusions reflect the species difference remains to be tested in the future.

Genetic approaches to peroxisome biogenesis

The genetic approach to identify components in the import machinery has been very fruitful (see review: Lazarow, 1993; Kunau

et al., 1993; Subramani, 1993). The strategy of this approach is straightforward: identify mutant organisms deficient in peroxisome import, rescue the peroxisome biogenesis of the mutants by transformation with a gene library, clone and characterize the wild type gene which effects the rescue. With this approach, several genes have been identified from human fibroblasts, CHO cells and different species of yeast.

Human genetic diseases: They result from the naturally occurring mutations in peroxisome biogenesis in the human population. Goldfischer and coworkers (Goldfischer et al., 1973) are the first to have observed that morphologically distinguishable peroxisomes were absent in liver and renal tubule cells of patients suffering from Zellweger syndrome. Since then, a broad spectrum of human genetic diseases has been found to be involved with the deficiency of peroxisome biogenesis (review, see Lazarow and Moser, 1989; Van den Bosch et al., 1992; Subramani, 1993).

Genetic diseases in humans involving peroxisome biogenesis can be tentatively classified into three groups depending on the degree to which peroxisomal functions are deficient. The first

category consists of Zellweger syndrome and two clinically somewhat milder diseases, the neonatal type of adrenoleukodystrophy (NALD) and the infantile type of Refsum disease. The biochemical abnormalities in this category are pleiotropic regarding the peroxisome function. Characteristics of the cell biology in cells from patients in this group are as follows: Most of the peroxisomal proteins are missing, though their synthesis is normal (Wanders et al., 1984; Wanders et al., 1985). Some peroxisomal proteins, such as catalase and some oxidases are mislocalized to the cytosol . Thiolase and nsLTP are not processed, existing only as their precursor forms (Lazarow et al., 1985; Wanders et al., 1985). There are no morphologically identifiable peroxisomes in most of the cells under routine electron microscopy. However, immunofluorescence using antibodies against peroxisomal membrane proteins identifies empty membrane structures (peroxisome "ghosts") (Santos et al., 1988; Santos et al., 1992). It is believed that diseases in this class are caused by mutations that result in the failure to import peroxisome proteins into the matrix. Complementation studies using somatic cell fusions define nine different groups, indicating that at least nine (Yajima et al., 1992;

Shimozawa et al., 1993) gene products are involved.

A second category is composed of two classes of diseases: the rhizomelic type of chondrodysplasia punctata (RCDP) and Zellweger-like syndrome. The characterization of diseases in this category is that only certain peroxisomal functions are deficient, while the peroxisome structure is normal in the cell from patients. In RCDP, plasmalogen synthesis is deficient, and thiolase is mainly present in the cytosol and not processed. Phytanic acid oxidation is also impaired. However, the ß-oxidation functions are much less affected, as is indicated by the fact that there is no accumulation of very long chain fatty acids in the cells from patients; Catalase is also present in peroxisomes (Heikoop et al., 1990; Naidu et al., 1988). In Zellweger-like syndrome, peroxisomal ß-oxidation enzyme proteins were found to be deficient upon immunoblotting, and DHAP-AT was deficient, whereas catalase was located in peroxisomes (Suzuki et al., 1988).

The third category includes a large number of disorders in which only single peroxisomal enzyme is deficient and in which normal peroxisomes can be found in tissues and cultured cells. One

example is the primary hyperoxaluria type I, which is the result of mutations in the peroxisomal alanine:glyoxylate aminotransferase (Purdue et al., 1990).

The studies of those genetic disease have supplied a large fund of knowledge about the biochemistry, function and cell biology of peroxisomes. The collection of fibroblasts from these patients are valuable resources for the cloning of genes. But technical difficulties have made the gene cloning in human fibroblasts very hard. The human fibroblasts are much more difficult to be immortalized and transfected as compared with rodent fibroblasts. Up to now only one human gene required for peroxisome biogenesis has been cloned by hybridization to a human cDNA library using rat PAF-1 as a probe (Shimozawa et al., 1992).

***Peroxisome biogenesis mutants from CHO cells*:** Raetz and coworkers first isolated three CHO mutants deficient in peroxisomes, using an autoradiographic screening procedure for the deficiency of DHAP-AT (Zoeller and Raetz, 1986). Later, Fujiki and coworkers isolated and characterized two mutants in CHO cells lacking peroxisomes using the same screening technique (Tsukamoto

et al., 1990). These two mutants did not complement each other. In mutant cells, catalase, acyl-CoA oxidase, and thiolase are mislocalized to the cytosol; similar to what is seen in Zellweger syndrome. By transfecting a rat cDNA library into one mutant cell line, the wild type gene was cloned and termed PAF-1 (Tsukamoto et al., 1991). PAF-1 encodes a peroxisomal membrane protein with a molecular weight of 35,000 kDa. Though the cellular function of this protein remains to be found, PAF-1 is a good candidate for a receptor or translocator.

The human homologue of PAF-1 was cloned by screening a human cDNA library with the rat PAF-1 cDNA (Shimozawa et al., 1992). The human and rat PAF-1 both are able to rescue peroxisome biogenesis in fibroblasts from one patient with Zellweger syndrome. This is the first successful cloning of a human gene required for peroxisome biogenesis.

Mutants in yeast: At the time this project began (May, 1990), Erdmann et al. (1989) had isolated three peroxisome assembly (*pas*) mutants in *Saccharomyces cerevisiae* based on the failure of mutant cells to grow on media using oleic acid as a sole carbon source . The

phenotype of the *pas* mutants is very similar to what is seen in Zellweger syndrome. Three mutants deficient in peroxisome assembly have been isolated in *Hansenula polymorpha* by two groups (Didion and Roggenkamp, 1990; Cregg et al., 1990). By this time, no yeast gene required for peroxisome biogenesis had been cloned by a genetic approach. Two genes encoding peripheral peroxisomal membrane proteins PMP20 (Garrard and Goodman, 1989) and one gene encoding an integral membrane protein PMP47 (McCammon et al., 1990a) in yeast *C. boidinii* have been cloned with a reverse genetic approach. The involvement of these proteins in peroxisome assembly, although possible, has not been tested yet. The studies of the isolation of yeast mutants deficient in peroxisome assembly and the cloning of the corresponding wild type genes have been very fruitful in the last 2-3 years. These works will be discussed in Chapter 5.

Aim of the thesis project

In this project, I intended to use *S. cerevisiae* as a model system to study peroxisome biogenesis with a genetic approach. This would include the development of an efficient selection strategy to isolate

peroxisome biogenesis mutants in this yeast, characterization of the isolated mutants, the cloning of a gene required for peroxisome biogenesis and the study of the gene product biochemically.

Chapter 2

Novel peroxisome clustering mutants and peroxisome biogenesis mutants of *Saccharomyces cerevisiae*

Introduction

The yeast, *S. cerevisiae*, has several advantages for studying peroxisome biogenesis. First, *S. cerevisiae* has peroxisomes, and the basic structure and function of peroxisomes in this yeast and mammalian cells is very similar. Mammalian peroxisomal proteins can be expressed in *S. cerevisiae* and imported into peroxisomes (de Hoop et al., 1993). This implies that the translocation machinery of peroxisomes in yeast and mammalian cells is similar. Secondly, the peroxisome in *S. cerevisiae* can be repressed by glucose and induced by oleate (Veenhuis et al., 1987, Wang et al., 1992). Thirdly, the classical and molecular genetics are well developed in this yeast. Lastly, yeast peroxisomes can be isolated by cell fractionation (McCammon et al., 1990b; Thieringer et al., 1991), and an in vitro

import assay has been established to study the translocation of newly synthesized proteins into the organelle (Thieringer et al., 1991). These features make S. cerevisiae an attractive model organism for analyzing peroxisome biogenesis.

Erdmann et al. have isolated several pas mutants in *S. cerevisiae* (Erdmann et al., 1989), but far fewer than the identified nine complementation groups of Zellweger patients in human beings (Shimozawa et al., 1993). This project was intended to use a genetic method to study the peroxisomal biogenesis in *Saccharomyces cerevisiae*. In this Chapter, I described the development of a positive selection method to isolate additional peroxisome biogenesis (*peb*) mutants and the characterization of these *peb* mutants with morphological methods.

Materials and Methods

Yeast Strains and Media

The yeast strains used in this study are described in Table 2-1. YPD, YPG, synthetic medium, synthetic minimal media with various nutrients and the sporulation medium were prepared as described by Sherman et al. (Sherman et al., 1986). YPOT was prepared according to Thieringer et al. (1991). YPGO contains 0.1% (w/v) oleic acid and 0.25% (v/v) Tween 40 added to YPG medium. YNO was prepared according to Erdmann et al. (1989). 2% (w/v) Bacto-agar was added to the appropriate media to make plates.

Immunofluorescence

The immunofluorescence procedure of Pringle et al. (1991) was simplified in order to analyze 50-100 clones

Table 2-1. Yeast Strains Used in This Study

Name	Genotype	Source
GC1-8B	*MATa, leu2-3,112, ura3-1, trp1-1, ctt1-1, cta1-2*	(Cohen et al, 1985)
DCT1-2C	*MATa, leu1, arg4, ctt1-1*	(Cohen et al., 1985)
JW68-3A	*MATα, ura3-1, trp1-1, arg4, ctt1-1*	this study
m6-D1	*MATa, peb1-1, ura3-1, trp1-1, ctt1-1*	this study
m11-A1	*MATα, peb2-1, ura3-1, trp1-1, arg4, ctt1-1,*	this study
m33-C2	*MATα, peb3-1, ura3-1, trp1-1, arg4, ctt1-1*	this study
2m1-A4	*MATα, peb4-1, leu2-3 112, ura3-1, trp1-1, arg4, ctt1-1*	this study
m34-A4	*MATa, peb5-1, leu1, trp1-1, arg4, ctt1*-1	this study
m24-C4	*MATα, peb1-2, trp1, ura3-1, ctt1-1*	this study
BQS20	*MATa, ura3-1, leu::HIS3, pot1::URA3*	(Igual et al., 1991)

daily. Cells were precultured in 2 ml of YPD overnight, inoculated into YPGO medium at a 1:50 dilution and grown for 18 to 20 h. The cells were fixed by the addition to the culture of 1/10 volume of 37% formaldehyde. The fixation time was typically 1 h at room temperature although times ranging from 20 minutes to 2 h were satisfactory. The cells were then washed twice with 100 mM phosphate buffer (pH 7.4) and once with SP buffer (1.2 M sorbitol, 20 mM potassium phosphate buffer, pH 7.4). Cell walls were digested with Zymolyase (10 μg/ml) in SP buffer containing 1 μl/ml β-mercaptoethanol at 30° C for 30 minutes. In most cases, about 70% of the cells were converted to spheroplasts. The spheroplasts were washed twice with SP buffer and resuspended in 30 μl of SP buffer. One μl of cell suspension was applied to a multi-well slide coated with poly-L-lysine (each well can hold 4-6 samples), and air dried for 5 minutes. Slides were immersed in methanol at -20°C for 5 minutes. The wells were washed 10 times with 50 μl of 1% BSA in PBS. The primary antibody (15 μl of a 200- to 1000-fold dilution) was applied to each well and incubated at room temperature for 2 h. Slides were further washed 10 times with 1% BSA in PBS. The fluorochrome-linked secondary antibody (500-fold dilution) was

applied and incubated for 1 h at room temperature. The cells were again washed 10 times with PBS. Mounting medium was added and cover glasses were applied and sealed with nail polish. Cells were observed under a Zeiss Axiophot fluorescence microscope.

Induction of Peroxisomes

The following standard conditions were used to induce peroxisomes in all experiments unless indicated otherwise. Cells were precultured in YPD medium overnight, diluted into more YPD medium at 3 x 10^4/ml and grown exponentially for 16 to 18 h to a final density of approximately 5 x 10^7 cells/ml. The cells were then inoculated into YPGO medium at a density of 5 x 10^6 and grown for 18 h at 30°C. Under these conditions, wild type cells, which have a doubling time of approximately 6 h in YPGO, were still growing exponentially. Cells were collected by centrifugation.

Electron Microscopy

Morphology. Whole cells were prepared as described by McConnell et al. (1990) and Stevens (1977) with the following modifications. Cells were prefixed with 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 1-2 h at 4°C. Fixation was continued with 4% potassium permanganate for 1 h. The cells were post-fixed in 2% osmium tetroxide, stained with 1.5% uranyl acetate for 2-12 h, dehydrated in ethanol and embedded in Epon 812. Fixation, dehydration and staining were performed on a rotator at room temperature. Sections were cut with a Reichert Ultracut E ultramicrotome and examined under a Hitachi 7000 electron microscope.

Cytochemistry for catalase activity. Whole cells were prefixed with 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 1 hour at 4°C (Erdmann et al., 1989) and incubated in 3, 3'-diaminobenzidine (DAB) (2 mg/ml) as described by van Dijken et al. (van Dijken et al., 1975). After the DAB reaction, cells were fixed with 1.5% potassium permanganate. The cells were then stained with 1.5% uranyl acetate and processed as described above. In control experiments, 50 mM 3-amino-1,2,4-triazole was included in

the DAB reaction to specifically inhibit catalase activity (Fahimi, 1969; Novikoff and Goldfischer, 1969).

Immunoelectron microscopic cytochemistry. Whole cells were processed as described by Slot and Geuze (1984) and by van Tuinen and Riezman (1987) with the following modifications. Cells were fixed as a suspension in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 2 h at room temperature. After washing, cells were incubated in 1% sodium metaperiodate for 1 h. Free aldehydes were quenched with 50 mM NH_4Cl. Small pellets were dehydrated in ethanol at -20°C, embedded in Lowicryl K4M and polymerized at -20°C under UV light for 24 hours. Silver sections were mounted on formvar coated 200 mesh nickel grids and blocked with 1% BSA in PBS. The sections were incubated with the primary antibody for 1 h, washed with PBS, and then incubated for 1 h in 10 nM gold-protein A (1:50). After the immunoreaction, the sections were further stained with uranyl acetate followed by lead citrate and examined by electron microscopy.

Other methods

Mutagenesis with 3% EMS was carried out (Sherman et al., 1986) with 60% mortality. Techniques of yeast genetics such as cell mating, sporulation and tetrad analysis were done according to the standard protocols of Sherman et al. (1986). Proteins were separated by SDS-PAGE and immunoblotted according to standard protocol (Harlow and Lane, 1988); antibodies on the blots were detected by chemiluminescence with a western blot detection kit from Amersham. Catalase was assayed as described previously (Baudhuin et al., 1964).

Materials

Rabbit anti-yeast thiolase was kindly provided by Dr. Wolf Kunau (University of Bochum, Germany). A rabbit antiserum against total peroxisomal proteins from *Candida tropicalis* (#10-324) (Small et al., 1987) cross-reacts with catalase and several peroxisomal proteins of unknown function in *S. cerevisiae* (G. M. Small, Mount Sinai School of Medicine, New York, personal communication). It

does not cross-react with *S. cerevisiae* thiolase. FITC-conjugated goat anti-rabbit Ig was from Boehringer Mannheim (Germany). Texas Red linked to sheep anti - rat Ig was from Amersham International plc (England). Monoclonal rat anti-yeast-α-tubulin (YOL1/34) was from Accurate Chemical and Scientific Corp. (Westbury, NY). Horseradish peroxidase-conjugated goat anti-rabbit Ig and enhanced chemiluminescence western blotting detection reagents were from Amersham International plc (England). Ethylmethane-sulfonate (EMS) was from Sigma (M 0880). Zymolyase 100-T was from ICN Biomedicals, Inc.

RESULTS

Strategy for Isolation of Peroxisome Biogenesis Mutants

In *S. cerevisiae*, fatty acid ß-oxidation occurs in peroxisomes, which proliferate when the yeast is grown in medium containing fatty acids (Veenhuis et al., 1987; Skoneczny et al., 1988). H_2O_2 is a by-product of this metabolism, one H_2O_2 being produced each time a fatty acid is shortened by 2 carbons. H_2O_2 is very toxic if it accumulates in the cell. It is mainly degraded by two isozymes of catalase: catalase A within the peroxisome and catalase T in the cytosol (Fig. 2-1). I hypothesized that if a yeast strain lacking both catalase A and catalase T were to be grown in a medium containing oleic acid, the H_2O_2 generated during the ß-oxidation of oleic acid might accumulate in the cell and cause DNA damage or other cytotoxicity. This could result in the retardation of cell growth or even cell death.

I further supposed that if the yeast strain lacking catalase activity were mutagenized, any mutants in which peroxisome

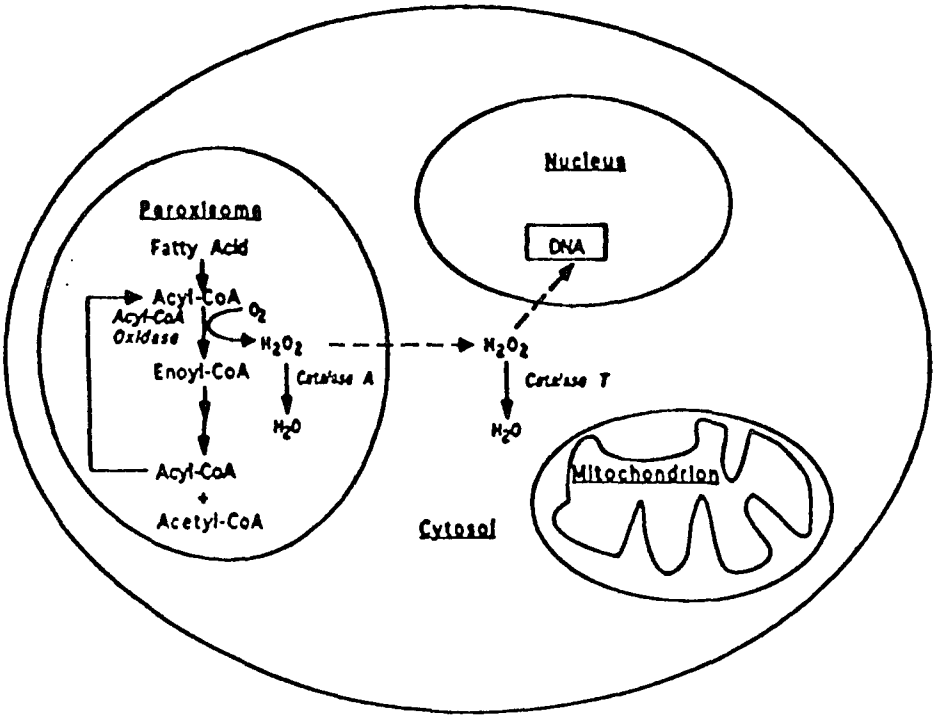


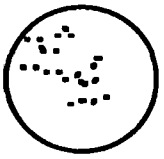
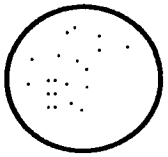
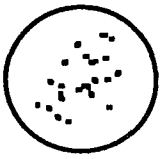
Figure 2-1. Protection against H_2O_2 toxicity in *S. cerevisiae*. H_2O_2 is formed in peroxisomes when a double bond is produced in a fatty acid undergoing ß-oxidative conversion to acetyl-CoA. The H_2O_2 is normally decomposed within the peroxisome by catalase isozyme A. Any H_2O_2 escaping from the peroxisome will be degraded by the cytosolic catalase isozyme T before it can damage other macromolecules, such as DNA.

biogenesis failed might be unable to carry out peroxisomal fatty acid ß-oxidation, and therefore would not produce H_2O_2 when grown in the presence of oleic acid. In the absence of H_2O_2 cytotoxicity, such mutants would be expected to grow normally, provided that an alternative carbon source were available. The normal-growing mutants could be readily identified and isolated for further analysis (Table 2-2). This would be the basis for a positive selection procedure.

Toxicity of H_2O_2 Produced During Peroxisomal Oleate Metabolism in Catalase-lacking Yeast.

I tested this hypothesis by experiments. Two related yeast strains, GC1-8B, which lacks both catalase A and catalase T, and DCT1-2C, which lacks only cytosolic catalase T (Cohen et al., 1985), were precultured in oleate-containing medium to induce peroxisomes and then were spread on plates containing oleic acid plus glycerol as a carbon source (YPGO). The strain devoid of catalase activity was viable, but grew very slowly, forming tiny colonies after 5 days (Fig. 2-2B). The strain containing peroxisomal catalase grew much faster

Table 2-2 Strategy to Isolate Peroxisome Biogenesis (*peb*) Mutants Based on the Toxicity of H_2O_2

	Yeast strains		
	Wild type	Catalase-deficient	peb mutant of catalase-deficient
Growth medium: carbon source	Glycerol and oleate	Glycerol and oleate	Glycerol and oleate
Fatty acid oxidation & production of H_2O_2	+	+	−
Catalase activity and decompositon of H_2O_2	+	−	−
Accumulation of H_2O_2	−	+	−
Expected growth	 Normal colonies	 Tiny or no colonies	 Normal colonies

and formed large colonies (Fig. 2-2A). Both strains grew rapidly on plates containing glycerol alone (YPG) (Table 2-3). This suggests that H_2O_2 formation is indeed toxic to cells lacking catalase.

The strain lacking catalase was mutagenized with 3% EMS, cultured for 24 h with oleate (YPOT), and spread on YPGO plates. Mutants unable to form peroxisomes were predicted to not make H_2O_2, and therefore to be healthy and grow III (Table I). As expected, a few large colonies were observed on a lawn of tiny colonies (Fig. 2-2C). These data suggested that the proposed positive selection procedure was feasible.

Isolation of Peroxisome Biogenesis (peb) Mutants

Approximately 1 x 10^6 mutagenized cells were screened for the ability to grow rapidly (on about 100 YPGO plates as in Fig. 2-2C). Nearly 1.7 x 10^3 large colonies were found on the background of numerous tiny colonies. These large colonies were candidates for *peb* mutants. I were aware, however, that large colonies could also have resulted from other mutations, such as structural defects in

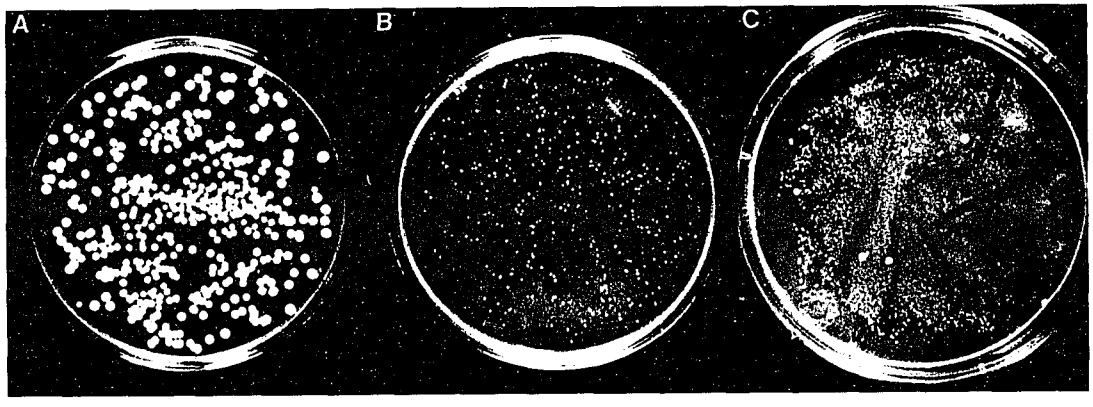


Figure 2-2. Growth of catalase-containing and catalase-deficient yeast in the presence of the fatty acid, oleate. (*A.*) Catalase A-containing strain (DCT1-2C) (*B.*) Catalase-deficient strain (GC1-8B). (*C*) Catalase-deficient strain after mutagenesis. The strains were precultured in YPD medium overnight, inoculated into YPOT medium at 1 x 10^6/ml, and grown for 24 hours. Cells were then spread on YPGO plates (which contain both glycerol and oleate) and incubated for 5 days at 30°C. About 400 cells were put on plates (*A)* and (*B*)and about 10^4 cells on plate (*C).* YPD preculture was omitted in (*C).*

Table 2-3 Growth of Yeast Strains with or without oleate in the Medium.*

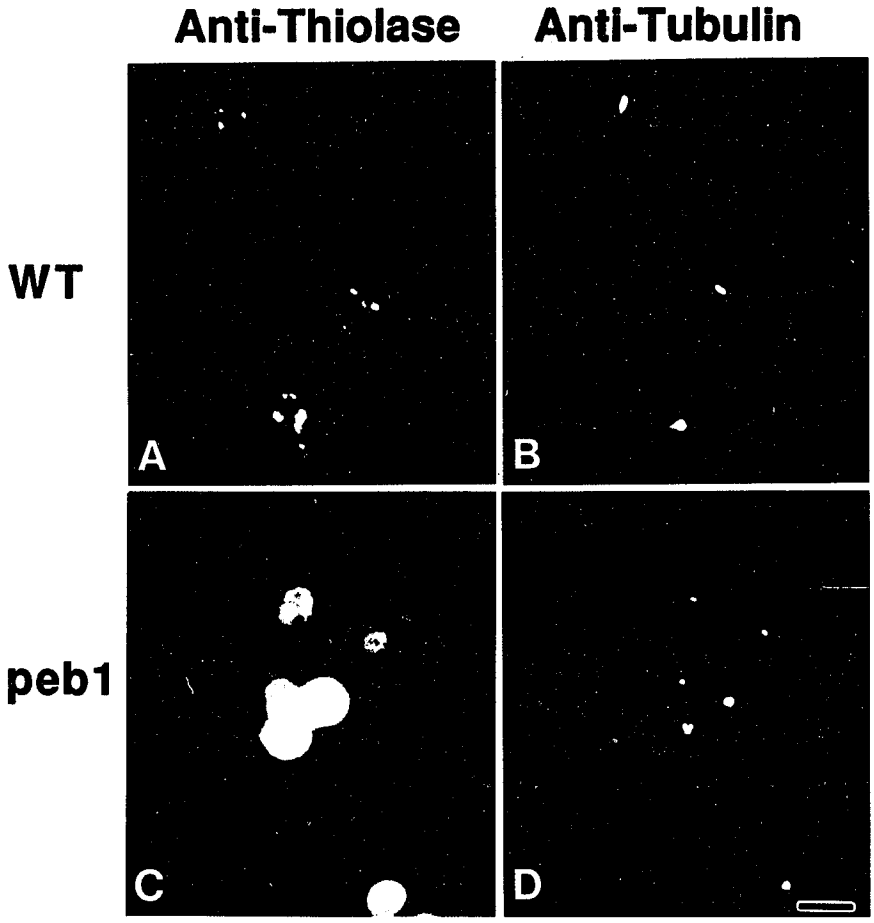
Yeast strain	DCT1-2C	GC1-8B
Cytosolic catalase T	No	No
Peroxisomal catalase A	Yes	No
Growth on glycerol	Large colonies	Large colonies
Growth on glycerol plus oleic acid	Large colonies	Tiny colonies

*The strains were precultured in YPD medium, grown for 24 hours in YPOT medium, and spread on YPG or YPGO plates. The plates were incubated for 5 days.

fatty acid oxidation enzymes. Therefore, a simplified immunofluorescence procedure (see Methods) was employed to screen the collection of large colonies for mutants which were deficient in peroxisome biogenesis. An antibody against thiolase, an enzyme that is normally located exclusively inside peroxisomes, was used for this purpose. It produced a punctate pattern of immunofluorescence in wild type cells containing normal peroxisomes (Fig. 2-3*A*).

Among the large colonies, 6.3% (107) had an abnormal thiolase fluorescence pattern. Some of these mutants showed thiolase throughout the cytosol (Fig. 2-3*C*). The fluorescence intensity of these cells appeared to be substantially greater than in wild type cells for unknown reasons. Subsequent immunoblot analysis showed that the total amount of thiolase protein in these mutants was not significantly different from wild type (see Chapter 3). Other mutant phenotypes of thiolase immunofluorescence are described below.

Figure 2- 3. Simplified double immunofluorescence of yeast cells in order to analyze thiolase with a tubulin control. Yeast colonies (50 - 100 at a time) were converted to spheroplasts, lightly fixed and permeabilized as described in Materials and Methods. A mixture of rabbit antiserum against yeast thiolase *(A, C)* and monoclonal rat antibody against yeast tubulin *(B,D)* was applied. The secondary antibodies were goat anti - rabbit IgG conjugated with FITC and sheep anti-rat IgG conjugated with Texas red. *(A, B),* wild type cells observed sequentially for thiolase *(A)* and tubulin *(B)*. *(C, D)* mutant cells, m6, observed sequentially for thiolase *(C)* and tubulin *(D)*. Bar = 5 μm.


Anti-Thiolase
Anti-Tubulin
WT
peb1
A
B
C
D

Cloning and Rescreening

Cells from each of the 107 large colonies with abnormal thiolase immunofluorescence patterns were cloned and reanalyzed by immunofluorescence at least twice. As a positive control, monoclonal antibodies against tubulin were included (Fig. 2-3*B, D*). 11 clones with reproducible and interesting mutant immunofluorescence phenotypes were chosen for further analysis.

The mutants were tested for their ability to grow on oleic acid as sole carbon source (YNO plates), which was expected to require intact peroxisomes. The mutants with altered intracellular distributions of thiolase (as assessed by fluorescence) did not grow at all on YNO, whereas the parent strain (GC1-8B), grew slowly (data not shown).

Genetic Analysis

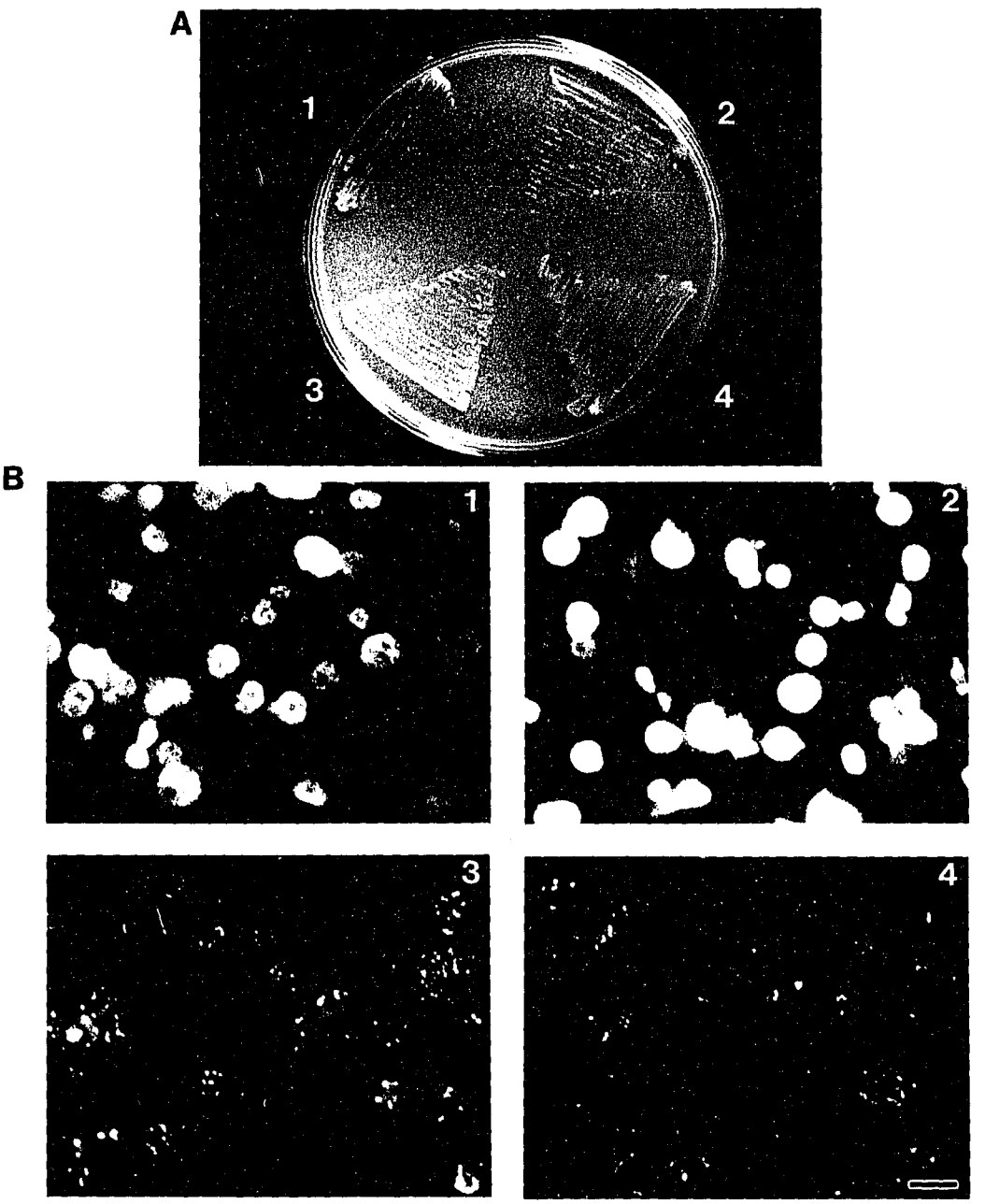
Each of the 11 mutant clones described above was mated with wild type yeast. Diploids were tested for their ability to grow on oleate as sole carbon source (YNO plates), and for peroxisome integrity (by

immunofluorescence). All diploid cells grew on YNO plates and had wild type thiolase fluorescence patterns (data not shown), indicating that the mutations in these 11 clones are recessive.

The diploids were sporulated and 10 to 12 tetrads from each cross were dissected. All showed a 2:2 segregation pattern for growth on YNO. Three or four tetrads per cross were further analyzed by immunofluorescence and all showed 2:2 segregation for punctate, wild type thiolase immunofluorescence. A typical result is illustrated in Fig. 2-4: two of the four meiotic products have cytosolic thiolase fluorescence and also fail to grow on YNO; the other two progeny have punctate immunofluorescence and do grow on YNO. These results show that each of the 11 mutant phenotypes is caused by a single gene mutation.

Peb mutants with appropriate mating types and genetic markers (selected from the tetrads) were used for complementation studies. Complementation was assessed by growth on YNO medium and by immunofluorescence. By both criteria, the 11 mutants fell

Figure 2-4. Genetic analysis of peroxisome biogenesis mutants. Mutant 2m1 was mated with wild type JW68-3A, diploid cells were sporulated, and tetrads were dissected. (*A*). Growth of the 4 spores from one tetrad on YNO plates. (*B*). Immunofluorescence of the same 4 meiotic progeny with antiserum against thiolase. Bar = 5 μm.


A
1
2
3
4
B
1
2
3
4

into five complementation groups (Table 2-4).

From each group, one mutant was chosen for further analysis. Mutant strains that contained catalase A were selected, in order that the packaging of this enzyme could be tested. The gene encoding catalase A came from the wild type strain with which the mutants had been backcrossed during the genetic analysis described above.

Immunofluorescence Patterns in the Five Peb Mutants

In the studies reported here and in the balance of this chapter, cells were routinely grown for 18 h in a medium containing both glycerol and oleic acid as carbon sources.

Peroxisomes were readily visualized by immunofluorescence with anti-thiolase in wild type yeast grown on YPGO. They appeared, in a typical focal plane, as 4-12 punctate fluorescence structures, usually located near the cell plasma membrane (Fig. 2-5). No appreciable cytosolic fluorescence was seen in wild type cells.

In three of the five new yeast mutant complementation groups

Mutants Lacking Recognizable Peroxisomes.

Table 2-4. Mutant Phenotypes and Complementation

Mutant	Thiolase immunolocalization pattern	Growth on YNO	*peb* Complementation group*
m6	cytosolic	-	1
m13	cytosolic	-	1
m24	cytosolic	-	1
m25	cytosolic	-	1
m28	cytosolic	-	1
m29	cytosolic	-	1
m11	cytosolic	-	2
m33	weak particles	-	3
m9	weak particles	-	3
2m1	cytosolic	-	4
m34	large particles	-	5

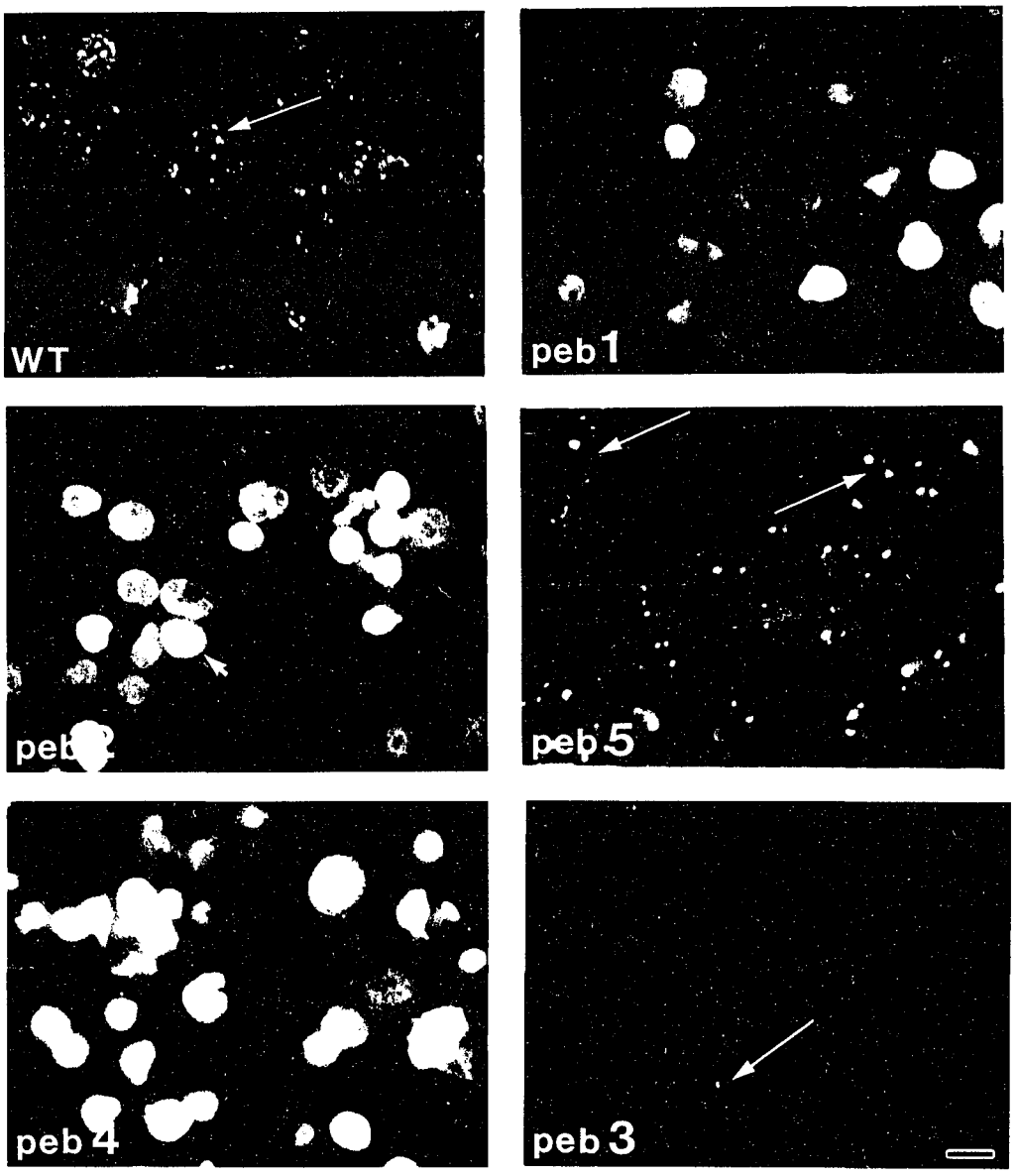
*All mutants were recessive and segregated 2:2 unless noted differently.

(*peb1*, *peb2* and *peb4*), the cell cytosol was filled with strong fluorescence, and no punctate structures were seen (Fig. 2-5). This indicates that thiolase was expressed in the mutants, but was not packaged into peroxisomes. The presence or absence of peroxisomes and the packaging of other peroxisomal proteins was examined by electron microscopy and immunolabeling, as described below.

In one new complementation group, *peb5*, most cells contained one or two strongly fluorescent structures, that were distinctly larger than normal peroxisomes (Fig. 2-5). These cells also contained some fluorescent particles with the size of normal peroxisomes. The huge fluorescent particles could be giant peroxisomes or clusters of peroxisomes or perhaps could be due to the mislocalization of thiolase into vacuoles.

In the *peb3* complementation group, cells contained punctate fluorescent structures, but the fluorescence intensity was much fainter than in wild type cells (Fig. 2-5). The number of particles also appeared to be less than that of wild type cells. This might

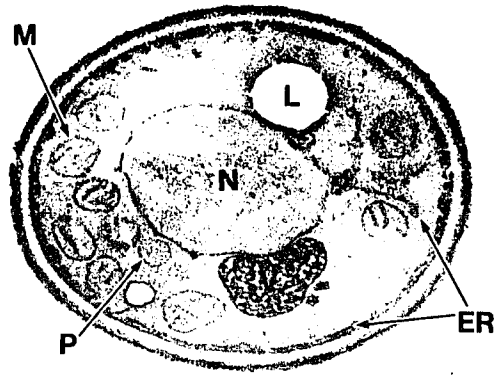
Figure 2-5. Immunofluorescence analysis of one mutant from each of the five *peb* complementation groups and of wild type cells (*WT*) with anti-thiolase. Arrows indicate cells with typical appearance, discussed in the text. In *peb1*, *peb2* and *peb3*, cells that appear to have little or no fluorescence are mostly above or below the focal plane. The yeast were grown in YPGO for 18 h. Bar = 5 μm.


WT
peb 1
peb 5
peb 4
peb 3

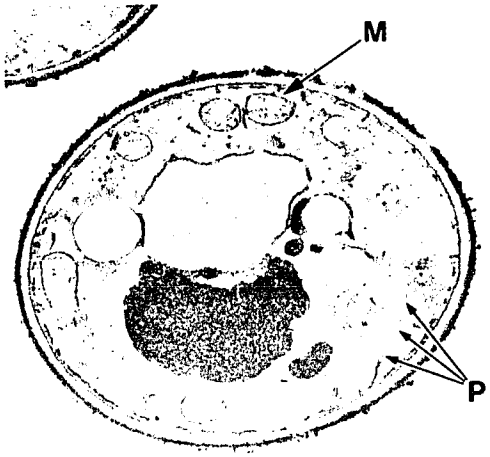
result from the reduced expression of thiolase, and perhaps of other peroxisomal proteins. Many other mutants detected in the initial screen showed this fluorescence phenotype; since it appeared likely that they represent regulation mutants, most of them were set aside for the time being.

Peb2 - electron microscopy. Mutant m11-A1 had a nearly normal appearance when grown on YPGO and examined by electron microscopy (Fig. 2-6, *peb2*). The nucleus, mitochondria and vacuole were present and demonstrated their usual ultrastructural appearances. Most of the ER was located adjacent to the plasma membrane. White (electron transmitting) droplets of lipid were frequently found in wild type and *peb2* cells. They result from the yeast having taken up fatty acid from the medium and stored it in droplets. No peroxisomes were found in *peb2* cells, despite careful examination of hundreds of sections. This contrasts with wild type cells grown in YPGO, in which peroxisomes were observed as individual, round structures scattered through the cytoplasm. In wild type yeast, the

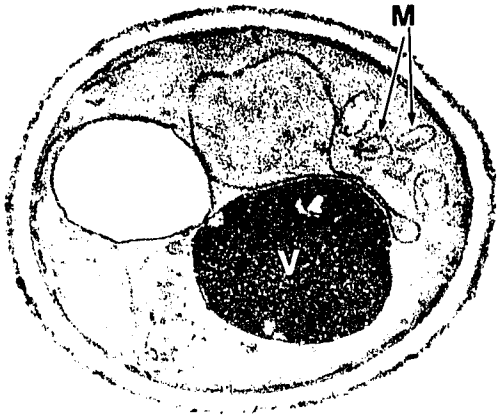
Figure 2-6. Electron microscopy of each of the five *peb* mutants and wild type cells (*WT*). *ER*, endoplasmic reticulum. *L*, lipid droplet. *M*, Mitochondrion. *N*, Nucleus. *P*, Peroxisome. *V*, Vacuole. Note the presence of clusters of peroxisomes in mutants carrying the *peb1-1* and *peb5-1* mutations. Growth in YPGO for 18 h. Bar = 0.4 μm.



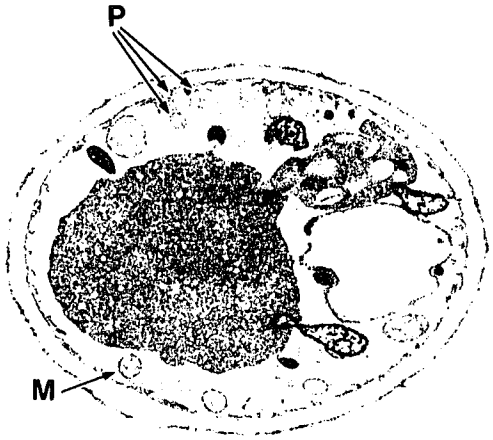
WT



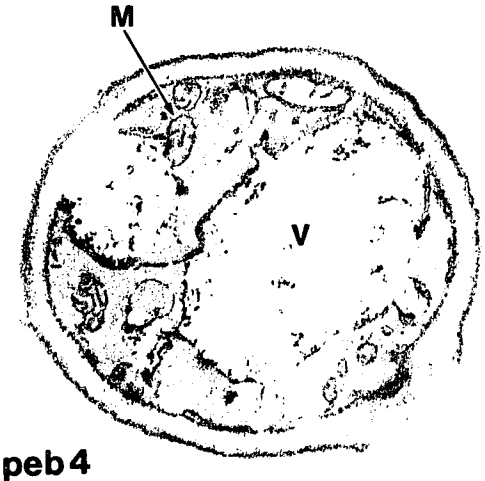
peb 1



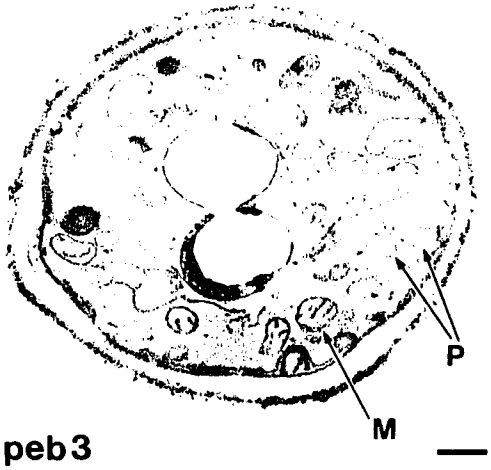
peb 2



peb 5

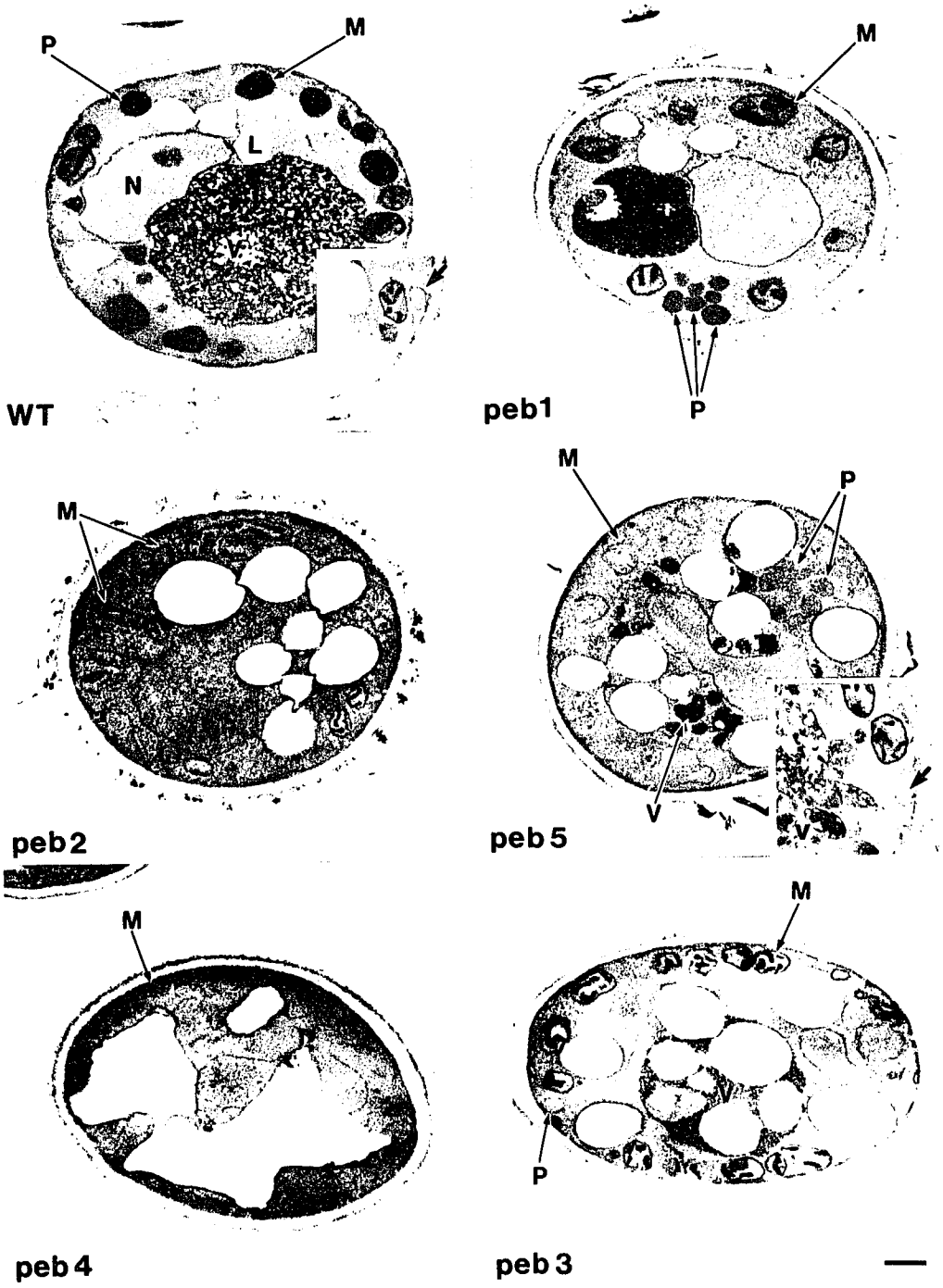


peb 4



peb 3

Figure 2-7. Cytochemical staining of catalase activity in the five *peb* mutants and wild type cells (*WT*). These strains contain a wild type catalase A gene which was introduced during the backcrossing of the mutants with wild type yeast. Electron-dense, oxidized diaminobenzidine is deposited on peroxisomes containing catalase, and also on mitochondrial cristae, due to an unrelated enzyme activity. Insets in (*WT*) and (*peb5*) show controls in which aminotriazole was included in the cytochemical reaction to specifically inhibit catalase activity; note the unstained peroxisomes (short arrows) and stained cristae. The intensity of cristae staining is lower in mutants in which there is a lot of cytosolic catalase, presumably because it prevents much of the externally-added H_2O_2 from reaching mitochondria. Abbreviations are the same as in Fig. 2-6. Growth was in YPGO for 18 h. Bar = 0.4 μm; same magnification in insets.


P
M
L
N
V
WT
M
P
peb1
M
peb2
M
P
V
V
peb5
M
peb4
M
P
peb3

peroxisomes were usually somewhat smaller than mitochondria (Fig. 2-6).

Peb2 - electron microscopic cytochemistry. In a further effort to find peroxisomes in *peb2* mutant cells, I employed a cytochemical reaction in which an electron-dense deposit of oxidized diaminobenzidine (DAB) is deposited on structures containing catalase (van Dijken et al., 1975). This causes wild type peroxisomes to stand out as darkly stained circles that are easy to identify (Fig. 2-7, WT). No peroxisomes were found in hundreds of sections of *peb2* cells (Fig. 2-7).

Catalase is present in *peb2* at approximately normal levels. It is probably mislocalized to the cytosol, where the concentration would be much low than within peroxisomes, which would explain the lack of demonstrable DAB staining. Cytosolic catalase in Zellweger hepatocytes likewise does not produce noticeable cytosolic DAB reaction product (Goldfischer et al., 1973).

peb4. Mutant 2m1, *peb4*, also lacked peroxisomes, based on electron microscopy (Fig. 2-6) and electron microscopic

cytochemistry (Fig. 2-7). The cytoplasm of this mutant consistently appeared abnormal, with large vacuoles and lipids droplets. Nevertheless, it grew as rapidly as *peb2*, other organelles such as mitochondria and ER were recognizable, and catalase activity was normal (see Chapter 3, Fig. 3-2). This mutant must be backcrossed further with wild type yeast in the future in order to test whether there is a connection between the absence of peroxisomes and the other morphological changes.

Peroxisome membrane ghosts? *Peb2* and *peb4* have a phenotype similar to Zellweger cells, in that normal-looking peroxisomes are missing. It has not yet been possible to test whether *peb2* and *peb4* cells have largely empty membrane ghosts of peroxisomes, such as are seen in Zellweger cells, because of the lack of a suitable antibody. However, one image of *peb4* showed curious membrane structures that could be candidates for peroxisome ghosts (Fig. 2-8). No such structures have been detected thus far in *peb2*.

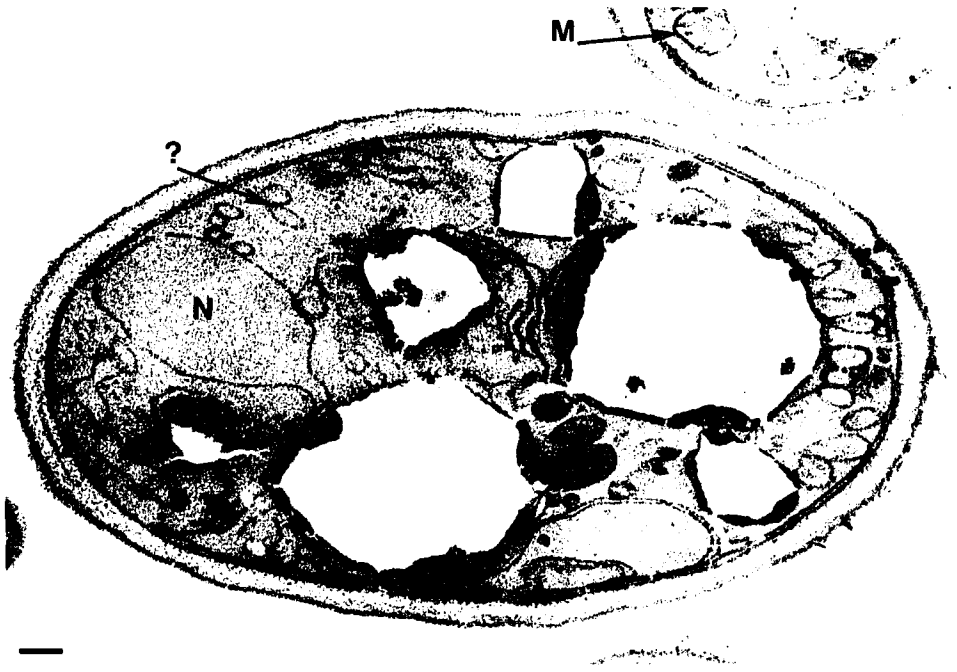


Figure 2-8. "Ghost" candidates (?) in *peb4*. Bar= 0.25 µm.

Complementation with Kunau's pas mutants. By this time, Kunau's group has isolated additional complementation groups of *pas* mutants. My *peb* mutant 2m1 was mated with each of the *pas* mutants *1, 2, 3, 4, 5* and *6* (Erdmann et al., 1989; Kunau and Hartig, 1992). In these six *pas* mutants, peroxisomal proteins, including thiolase, are mislocalized to the cytosol. In each mating, the resulting diploids showed wild type punctate thiolase fluorescence. Therefore, *peb4* belongs to a different complementation group than *pas1* to *6*. On the other hand, mutant m11 (*peb2*) failed to complement *pas1*, suggesting that m11 is an allele of *pas1*.

Mutants in Which Peroxisomes are Present in Clusters and Packaging of Peroxisomal Enzymes is Partially Defective.

peb1. Mutant m6-1, *peb1*, showed strong cytosolic fluorescence with anti-thiolase, just like *peb2* and *peb4*. However, *peb1* contained normal-looking peroxisomes by electron microscopy (Fig. 2-6). Instead of being distributed throughout the cytosol, the peroxisomes occurred in clusters. An example of a cluster of four

peroxisomes in *peb1* is shown in Fig. 2-6. Although clusters of peroxisomes were occasionally found in wild type yeast grown in YPGO, clustering was consistently observed in *peb1*.

Catalase is packaged normally into peroxisomes in *peb1*, as demonstrated by cytochemical staining. A cluster of seven DAB-positive peroxisomes is illustrated in Fig. 2-7 (*peb1* panel).

The inability of this mutant to package thiolase into peroxisomes, shown by the immunofluorescence data, was tested further by immunogold labeling. As shown in Fig. 2-9, wild type yeast peroxisomes, reacted with anti-thiolase, contained abundant gold particles (arrows). In contrast, peroxisomes in *peb1* were unlabeled. Thus this mutant has a partial defect in peroxisomal protein packaging: the clustered peroxisomes can import catalase but not thiolase.

Thiolase targeting information is intact in peb1.

The inability of the *peb1* mutant to package thiolase could be the

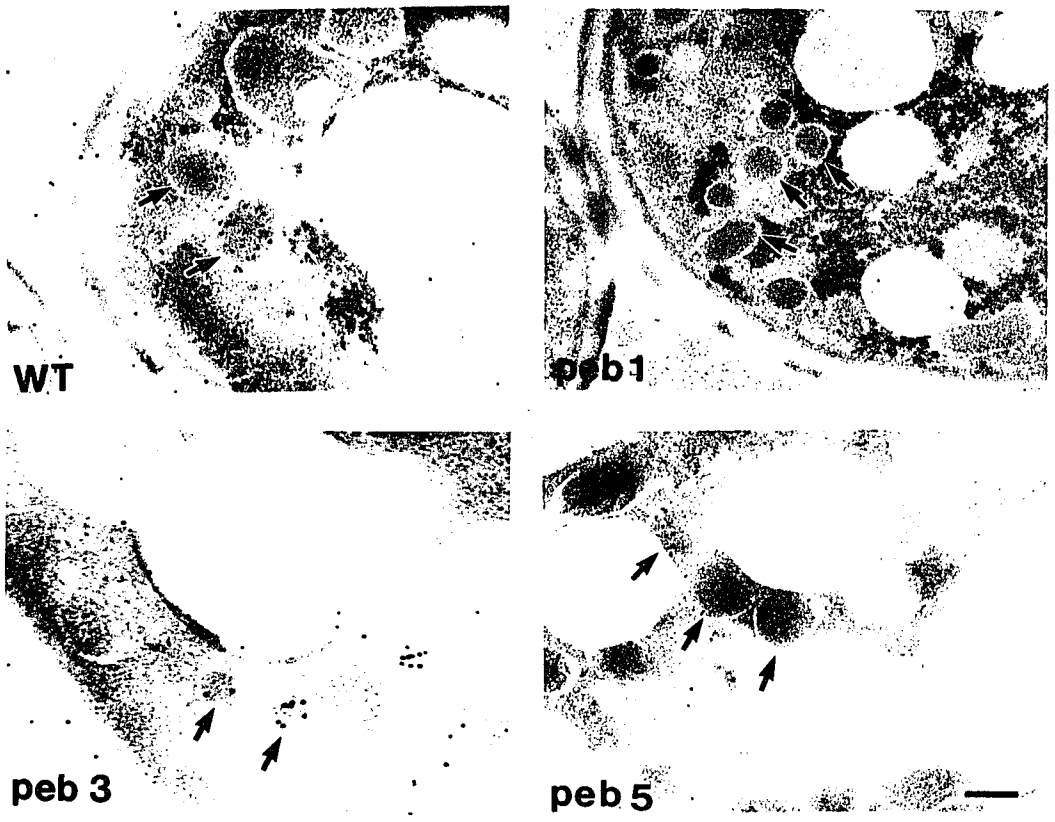
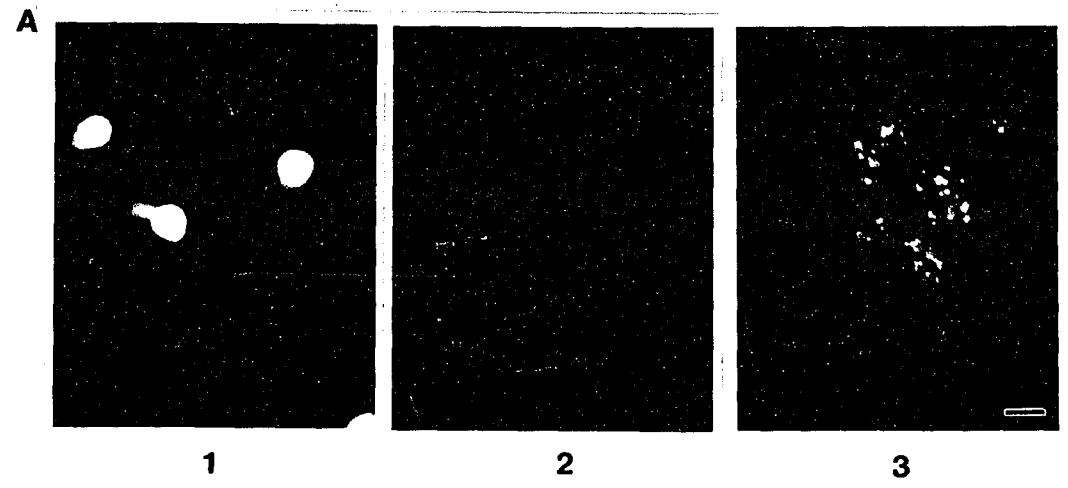
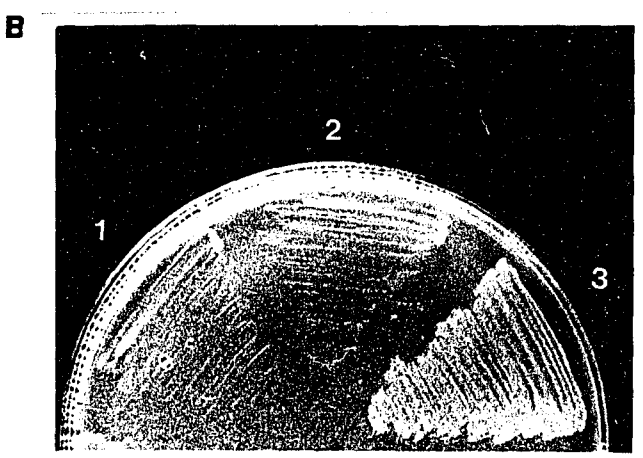


Figure 2-9. Immunoelectron microscopy to detect thiolase in wild type cells and in the three *peb* mutants in which peroxisomes were found. Sections were incubated with rabbit antiserum against thiolase, followed by gold-conjugated protein A. Arrows indicate peroxisomes. Bar = 0.2 μm.

Figure 2-10. Thiolase in the *peb1-1* mutant has functional targeting information. *Peb1-2* (m24-C2) (1) was mated with strain BQS20 in which the thiolase gene had been knocked out (*2*) and diploids (*3*) were selected. *(A).* Immunofluorescence analysis with antithiolase. (*B.*) Growth on YNO plates. (*C*) Immunoblot analysis with antithiolase. (*1*), *peb1-1* mutant. (2), thiolase knockout. (*3*), the diploid.


A
1
2
3


B
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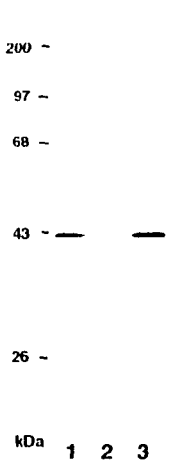

C
200
97
68
43
26
kDa
1 2 3

result of a mutation in the thiolase gene that abolishes the targeting information that directs thiolase to peroxisomes. Alternatively, it could be due to a mutation in a gene that encodes a protein that is required to import thiolase, but not catalase, into peroxisomes. These possibilities were tested by mating *peb1-2* with a yeast strain in which the thiolase gene had been knocked out (Igual et al., 1991). In the thiolase knockout strain, no thiolase protein was detected by immunoblotting (Fig. 2-10C, lane 2) or by immunofluorescence (Fig. 2-10A, panel 2). Like *peb1*, this strain did not grow on YNO (Fig. 2-10B, sectors 1 and 2). The diploid resulting from the cross between this strain and *peb1-2* demonstrated a wild type pattern of thiolase immunofluorescence (Fig. 2-10A, panel 3). Moreover, the diploid grew on YNO (Fig. 2-10B, sector 3), indicating regain of peroxisomal function. These data demonstrate that the thiolase in *peb1* contains the necessary targeting information to be packaged into peroxisomes. Therefore, *peb1* must contain a mutation in machinery that is specifically necessary for thiolase import.

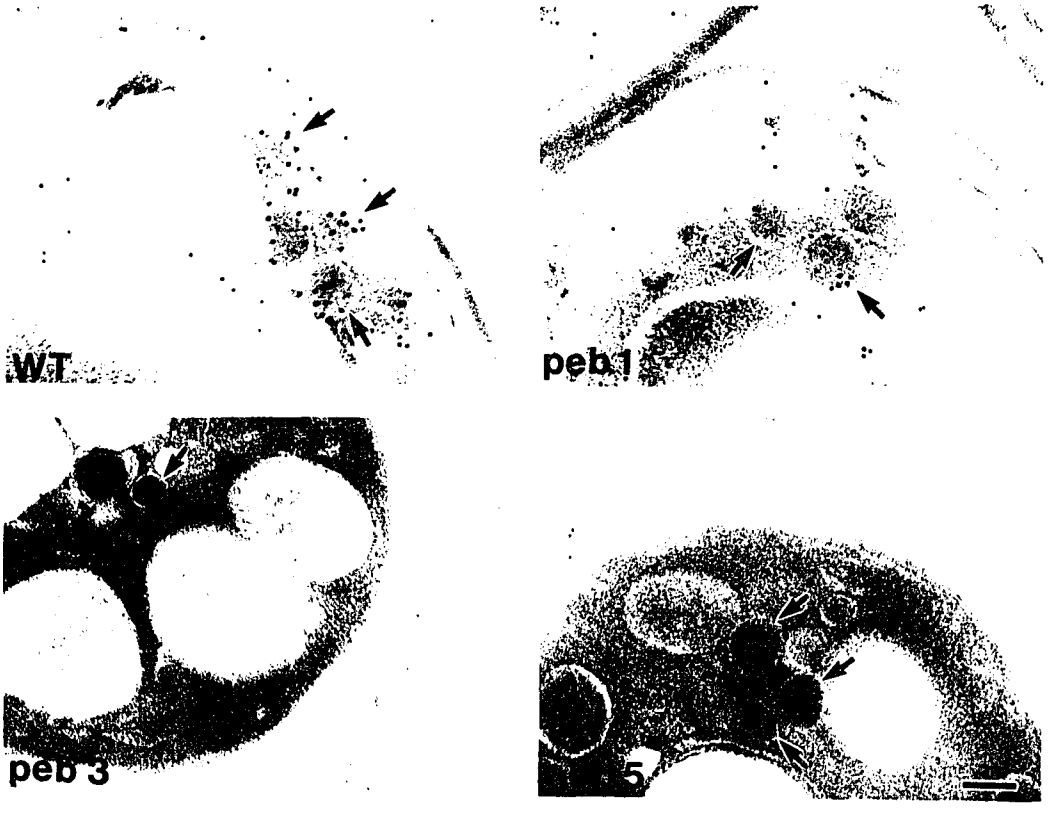


Figure 2-11. Immunoelectron microscopy with a rabbit antiserum that reacts with several peroxisomal proteins (see Methods) followed by gold-conjugated protein A. The cell samples are the same as in Fig. 2-9. Arrows indicate peroxisomes. Bar = 0.2 μm.

Immunoelectron microscopy was also carried out with an antibody that recognizes several peroxisomal proteins other than thiolase (see Methods). It gave a strong gold labeling of wild type peroxisomes, and a reduced labeling of peroxisomes in *peb1* (Fig. 2-11). This suggests that thiolase is probably not the only peroxisomal enzyme whose packaging is impaired in *peb1*.

peb5. In mutant m34, *peb5*, thiolase appeared to be in huge structures according to the immunofluorescence data (Fig. 2-5). By EM analysis, peroxisomes of normal size were present in *peb5*. As in *peb1*, these peroxisomes were consistently observed in clusters (Fig. 2-6). Immunoelectron microscopy of these peroxisomes demonstrated that they contain thiolase (Fig. 2-9). Therefore, the large fluorescent particles seen in Fig. 2-5 are in fact clusters of normal-sized peroxisomes.

These clustered peroxisomes do not contain catalase, according to EM cytochemistry. A cluster of 7 DAB-negative peroxisomes is shown in Fig. 2-7 (*peb5* panel). It is noteworthy that electron dense DAB was observed in vacuoles in *peb5*. However, this DAB deposition was also seen in cytochemical

controls in which catalase activity was inhibited with aminotriazole (Fig. 2-7, insert). Therefore, the vacuolar DAB is due to some cause other than catalase enzyme activity. Similar staining of mammalian lysosomes by DAB in a catalase-independent, non-enzymatic fashion has sometimes been observed (Fahimi, 1969; Novikoff and Goldfischer, 1969).

The activity of catalase in *peb5* was approximately the same as in wild type cells. The absence of DAB reactivity in the peroxisomes or in any other organelle in *peb5* suggests that the catalase is cytosolic. Mislocalization of catalase to the cytosol may also explain the reduced staining of mitochondrial cristae by DAB that was observed in *peb5*. The cytosolic catalase probably reduces the amount of externally added H_2O_2 that reaches the mitochondria during the cytochemical reaction. This interpretation is supported by the fact that when catalase was inhibited with aminotriazole, mitochondrial staining appeared (Fig. 2-7, inset to *peb5* panel).

A wild type catalase gene had been introduced into this *peb5* mutant by backcrossing. Therefore the inability to import

catalase into peroxisomes must be due to a defect in catalase-specific import machinery.

Immunogold labeling of peroxisomes with the antibody against several peroxisomal proteins was strikingly reduced in *peb5* relative to wild type (Fig. 2-11), suggesting that the packaging of additional proteins may also be impaired.

Both mutants m6 and m34 complemented all of the *pas* mutants *1* through *6*. Therefore, *peb1* and *peb5* are different complementation groups, as might be expected from the different phenotypes.

A Mutant in Which the Peroxisomes Contain Less of Several Proteins.

peb3. Mutant m33, *peb3*, showed weak fluorescent particles with anti-thiolase (Fig. 2-5). This mutant contained peroxisomes (Fig. 2-6), which appeared to be somewhat less abundant than in wild type cells. They did not show demonstrable DAB staining for catalase (Fig. 2-7). By immunoEM, they demonstrated reduced immunoreactivity for thiolase (Fig. 2-9) and no immunoreactivity

with the antibody against several other peroxisomal proteins (Fig. 2-11). The abundance of these enzymes was low, according to immunoblot analyses, described in the Chapter 3. Thus, *peb3* may be caused by a reduced expression of peroxisomal proteins (see Discussion of Chapter 3).

Discussion

The positive selection procedure used here, which exploited the toxicity of hydrogen peroxide, led to the identification of 5 *peb* complementation groups. Four of these are distinct from all of the previously described peroxisome assembly mutants, *pas1* through *6*, of Kunau (Erdmann et al., 1989, Kunau and Hartig, 1992). This illustrates the rule that different selection strategies often yield different mutants. In the present case, the difference in strategy was subtle: Kunau screened for mutants that were unable to utilize fatty acid as sole carbon source, whereas I selected against mutants that utilized fatty acid in the presence of glycerol. Van Der Leij et al. (1992) recently reported the isolation of some peroxisome assembly mutants by a variant of this procedure in which catalase was inhibited. There has not yet been an opportunity to cross-complement the mutants of the two labs. None of their mutants show the peroxisome clustering that I have observed, and our mutants did not have the reticular structure found in theirs.

Three of our complementation groups consisted of one

mutant each, clearly suggesting that the search for peroxisome biogenesis mutants in *S. cerevisiae* is still not completed. The simplified immunofluorescence procedure introduced here makes it possible to rapidly look for additional mutants in which individual peroxisomal proteins are incorrectly packaged. With the fluorescence screen one can avoid mutants in which the expression of peroxisomal proteins appears to be down-regulated which, while interesting, are not the focus of our current interest.

One of the most interesting aspects of our results is the unexpected observation that peroxisomes occur in distinct clusters in two of the mutants. There are many possible explanations. One speculation is that these peroxisomes might actually be interconnected. New peroxisomes form by division from preexisting peroxisomes (Lazarow and Fujiki, 1985), and if a protein that is required to finish the process of pinching off daughter peroxisomes were missing, the result might be an interconnected cluster, looking like a bunch of grapes. I plan to investigate this possibility in the future by serial sectioning.

Another unexpected result was the finding that two of the

mutants were partially defective for the import of peroxisomal proteins. *Peb1* can import catalase but not thiolase. *Peb5* can import thiolase but not catalase. These partial packaging defects may be related to the existence of multiple types of targeting information that direct proteins to peroxisomes. As being discussed in Chapter 1, at least 3 different targeting signal have been used for the import of peroxisomal proteins. *S. cerevisiae* catalase A uses a carboxyl-terminal SSNSKF which is similar to the SKL targeting sequence, in addition to an internal sequence, for its import into peroxisomes (Kragler et al., 1993). It is not yet known what kind of targeting information is used by *S. cerevisiae* thiolase, but if I assume that in *S. cerevisiae*, thiolase and catalase are directed to peroxisomes by different classes of targeting information, then I may speculate that specific receptors for these targeting sequences might be individually mutated. In *peb1* a receptor used by thiolase might be defective. In *peb5,* a receptor used by catalase might be defective. An *in vitro* import assay for *S. cerevisiae* peroxisomes (Thieringer et al., 1991) is available, with which these hypotheses may be tested.

It is noteworthy that *peb1* and *peb5* have different partial packaging defects, but both show peroxisome clustering. Since these are both due to single gene defects, I speculate that there must be two independent proteins that are required for pinching off new peroxisomes: one would have a targeting signal in common with catalase and the other a targeting signal in common with thiolase.

Two of our yeast mutants resemble human Zellweger syndrome in that recognizable peroxisomes are not detectable. In all of the 9 Zellweger complementation groups that have been identified to date, fibroblasts contain peroxisomal ghosts (Santos et al., 1988; Santos et al., 1992; Wiemer et al.,1989; Suzuki et al., 1992); thus these are import mutants. If our yeast mutants contain ghosts, analysis of the defective gene products should shed light on the process by which proteins are imported into peroxisomes. If the yeast mutants lack ghosts, they may shed light on the manner of assembly of the peroxisomal membrane itself.

Chapter 3

Three peroxisome protein packaging pathways suggested by selective permeabilization of yeast mutants defective in peroxisome biogenesis

Introduction

In Chapter 2, I have reported the isolation of new yeast mutants in which peroxisome biogenesis is defective ("*peb*" mutants). In two complementation groups, *peb2* and *peb4*, peroxisomes were not found at all by electron microscopy nor by EM cytochemistry (Chapter 2). These yeast mutants may be models of the human genetic disease, Zellweger syndrome, in which defects in peroxisome biogenesis result in the absence of morphologically recognizable peroxisomes

(Lazarow and Moser, 1989). In one complementation group, *peb3*, only a few peroxisomes, which displayed weak thiolase immunofluorescence, were detected.

Remarkably, in two mutant complementation groups, normal-looking peroxisomes were present, but they occurred in clusters. These peroxisomes contained many, but not all, of the normal peroxisome proteins. The two groups differed in which of the peroxisomal proteins was missing according to electron microscopic cytochemistry and immuno-gold labeling. In mutant *peb5-1*, catalase was not detected in peroxisomes but thiolase was present. In another complementation group, *peb1*, containing several mutants, the reverse was true.

I have now investigated in more detail, using cell fractionation, the intracellular locations of several peroxisomal proteins in our *peb* mutants. These proteins were selected because it was thought likely that they might have different topogenic signals. Catalase A, the peroxisomal catalase isozyme in *S. cerevisiae*, has an SKL-like C-terminus (SSNSKF) plus internal topogenic information (Kragler et al., 1993). SKL and related

tripeptides are responsible for targeting many proteins to peroxisomes (Gould et al., 1990) in a variety of species (Gould et al., 1990). Thiolase is directed to peroxisomes by a cleavable N-terminal peptide, at least in rat liver (Osumi et al., 1991; Swinkels et al., 1991). Acyl-CoA oxidase in *S. cerevisiae* contains neither an SKL-like C-terminus nor a thiolase-like N-terminus. I speculated that it might be targeted to peroxisomes by internal peptides, as it is in *C. tropicalis* (Small et al., 1988). As controls, the intracellular distributions of several enzymes belonging to other cell compartments were also analyzed.

In order to obtain reliable data, I had to overcome a drawback of current fractionation procedures for studying yeast peroxisomes. The problem was that peroxisomes are fragile and tend to be damaged during homogenization, centrifugation, and resuspension of fractions (McCammon et al., 1990b; Thieringer et al., 1991), causing leakage of the contents. Therefore, a more gentle procedure was sought.

In the case of animal cells, plasma membranes and intracellular membranes can be selectively permeabilized by

digitonin, based on their content of cholesterol (de Duve, 1965; Wanders et al., 1984). Digitonin forms a stoichiometric complex with cholesterol, producing holes in the membrane. The concentration of digitonin needed to permeabilize a membrane is inversely proportional to the abundance of sterol in that membrane. The plasma membrane, which is richest in cholesterol (Colbeau et al., 1971), is permeabilized by low concentrations of digitonin; this releases cytosolic proteins from the cells. The lysosome membrane contains less cholesterol and requires a higher concentration of digitonin to allow the lysosomal enzymes to leak out. The mitochondrion and peroxisome, which contain little if any cholesterol in their membranes, require the highest concentrations of digitonin (de Duve, 1965; Wanders et al., 1984); in this case the digitonin may be functioning as a nonspecific detergent. Thus, the intracellular locations of enzymes can be determined by analyzing the extent of their release from cells as a function of the digitonin concentration applied. An important advantage of this method is that it avoids mechanical breakage of the organelles, and the attendant artefactual release of organelle proteins.

S. cerevisiae does not contain cholesterol in its membranes, but does contain a related sterol, ergosterol (Longley et al., 1968; Rattray et al., 1975). I speculated that digitonin might also form complexes with ergosterol and thus permeabilize yeast membranes. Here I report the successful use of digitonin for the selective permeabilization of yeast cell membranes. I have analyzed the specificity of the protein packaging defects in our *peb* mutants, and found evidence for a three-pronged pathway of protein import into peroxisomes.

Materials and Methods

Digitonin titration

Cells were harvested by centrifugation at 1500 rpm for 5 min. The cell mass was weighed and washed twice with buffer A (100 mM TrisCl, pH 7.4; 50 mM EDTA). The cells were resuspended in 50 ml of buffer A containing 10 mM ß-mercaptoethanol and incubated at 30°C for 20 min with shaking. The cells were pelleted by centrifugation, resuspended in 6 volumes (of cell mass) of SP buffer (1.2 M sorbitol, 20 mM potassium phosphate buffer, pH 7.4) plus Zymolyase 100-T, and incubated at 30°C for 45 minutes with gentle shaking (60-100 rpm). For wild type cells, 2 mg of Zymolyase/gram cell mass was used. Some mutants required less Zymolyase and shorter times for good cell wall digestion. As little as 0.5 mg/g cell mass and 10 minutes of incubation was used in some cases. The formation of spheroplasts was monitored carefully under the microscope. When 80-90% of the cells had been converted to spheroplasts, the digestion was stopped by the addition of an equal volume of ice-cold SP buffer. They were harvested by centrifugation at 1700 rpm for 5 minutes at 4°C and washed twice with 10 volumes of ice-cold SP

buffer. The spheroplasts were then resuspended in 5 volumes of ice-cold SP buffer containing a mixture of protease inhibitors (0.7 mM each of chymostatin, antipain, pepstain, and leupeptin, and 20 μg/ml PMSF). Ethanol (0.1%) was added to protect catalase activity (Chance, 1950).

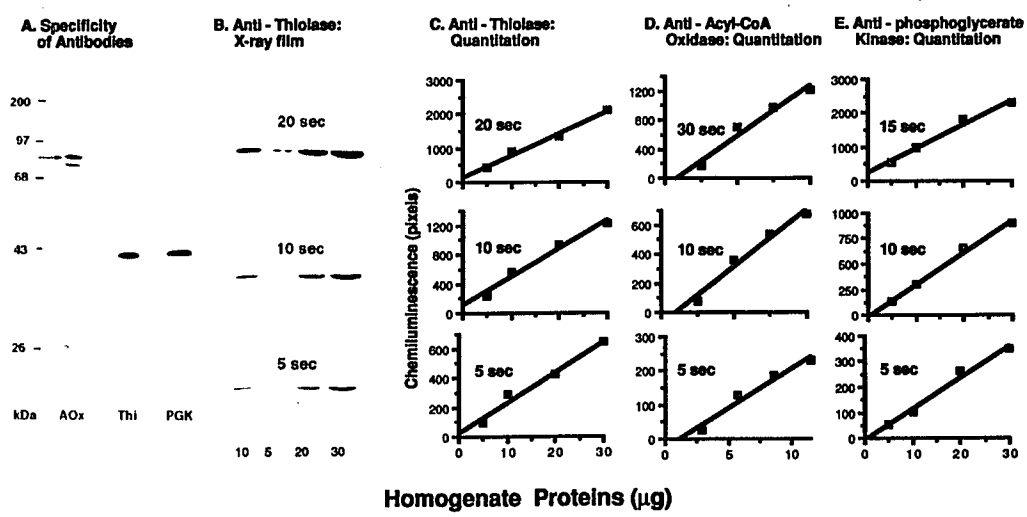
Aliquots of the spheroplast suspension (900 μl) were added to a series of tubes containing digitonin dissolved in 100 μl of SP buffer. The amounts of digitonin were chosen to obtain the final concentrations shown in Fig. 2-2*B*. The tubes were mixed gently by inversion, and incubated at 4°C for 20 min with rotation. After the incubation, the mixture was centrifuged in an Eppendorf microcentrifuge at 14000 rpm for 15 minutes at 4°C. The supernatants were recovered and were ready for further analysis.

Total cell homogenates were prepared by adding approximately 400 mg of glass beads to a tube containing 900 μl of yeast spheroplast suspension, vortexing for 5 minutes at 4°C, and centrifuging for 5 minutes at 5000 rpm. The supernatants were collected for analysis.

Western blots

Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes according to standard methods (Harlow and Lane, 1988). The Enhanced Chemiluminescence (ECL) western blotting system from Amersham was used for the immuno-detection of proteins according to the company's protocol. X-OMAT film from Kodak was used to record the signal. The films were scanned and converted to digitalized images with a Personal Densitometer (Molecular Dynamics). Scanning was conducted with 12-bit A/D converters to permit accurate quantitation (Nelson and Wygant, 1992) of the two-dimensional images. The integrated protein band intensities were computed with the ImageQuant software of the Personal Densitometer. In preliminary experiments, I found conditions where the signal was proportional to the antigen concentration over a reasonable range for each of the three

Figure 3-1. Quantitative detection of proteins by chemiluminescent immunoblotting. (*A*) Specificity of antibodies. Yeast extracts (20 μg of protein of total cell homogenates) were separated by SDS-PAGE, blotted with antisera against acyl-CoA oxidase (AOx), thiolase (Thi) and phosphoglycerate kinase (PGK). The antibodies were detected by chemiluminescence, which was recorded on X-ray film, as described in Materials and Methods. Arrow indicates AOx. (*B*) Effect of the amount of homogenate protein (5-30 μg) and the exposure time on the chemiluminescent signal from anti-thiolase. Digitized image of scanned film, printed on gray-scale printer. (*C*), (*D*) and (*E*) Quantitation of the chemiluminescence intensity by laser densitometry of the X-ray film and computerized integration of band densities. (*C*) Quantitation of thiolase data of panel B. (*D*) Acyl-CoA oxidase quantitation. (*E*) Phosphoglycerate kinase quantitation. Chemiluminescence exposure times are indicated in the figure.


A. Specificity of Antibodies
B. Anti - Thiolase: X-ray film
C. Anti - Thiolase: Quantitation
D. Anti - Acyl-CoA Oxidase: Quantitation
E. Anti - phosphoglycerate Kinase: Quantitation
200
97
68
43
26
kDa
AOx
Thi
PGK
20 sec
10 sec
5 sec
10 5 20 30
Chemiluminescence (pixels)
Homogenate Proteins (µg)

antibodies (anti-thiolase, anti-acyl-CoA oxidase, and anti-phosphoglycerate kinase) (Fig. 3-1). This method had another advantage in the case of the antiserum against acyl-CoA oxidase, which reacted with two minor bands in addition to the oxidase (Fig. 3-1*A*): it was easy to quantitate just the major band. These linear immunoblotting assay conditions were used in all of the experiments reported here. The immunoblot images were printed on an XL7700 continuous tone printer (Kodak) or Tektronix (Computer Graphic Technologies, NY, NY).

Assays

Fumarase (Bergmeyer et al., 1983a) and glyceraldehyde-3-phosphate dehydrogenase (Bergmeyer et al., 1983b) were assayed as described. Catalase was measured according to Baudhuin et al. (Baudhuin et al., 1964). Protein concentrations were determined by the Bio-Rad dye-binding assay with bovine immunoglobulin as a standard (Bradford, 1976).

Materials

Rabbit antiserum against *S. cerevisiae* acyl-CoA oxidase was a gift from Dr. Joel Goodman (University of Texas). Rabbit anti-yeast phosphoglycerate kinase was a gift from Dr. Jeremy Thorner (University of California, Berkeley). Digitonin was from Sigma (D1407, approximately 50% by TLC analysis according to the company; in this study, it was assumed to be 100%). Glycerol-3-phosphate was from Boehringer Mannheim (Germany). Molecular mass standards were from GIBCO, BRL. Zymolyase 100-T was from ICN Biomedicals, Inc.

Results

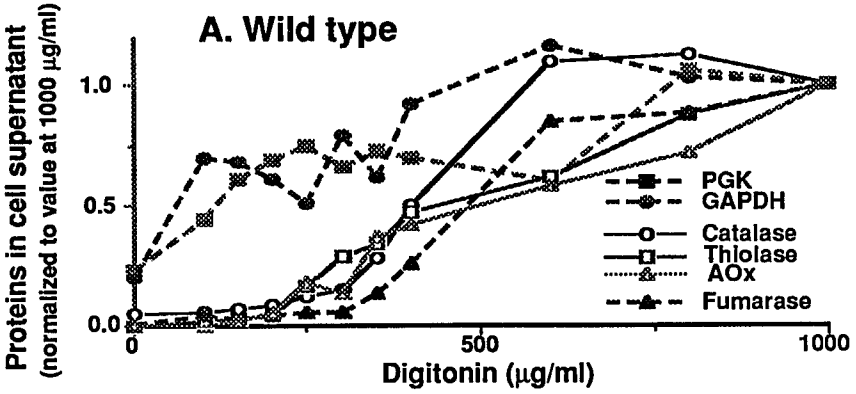
Cell Fractionation of S. cerevisiae by Selective Permeabilization of Its Membranes With Digitonin.

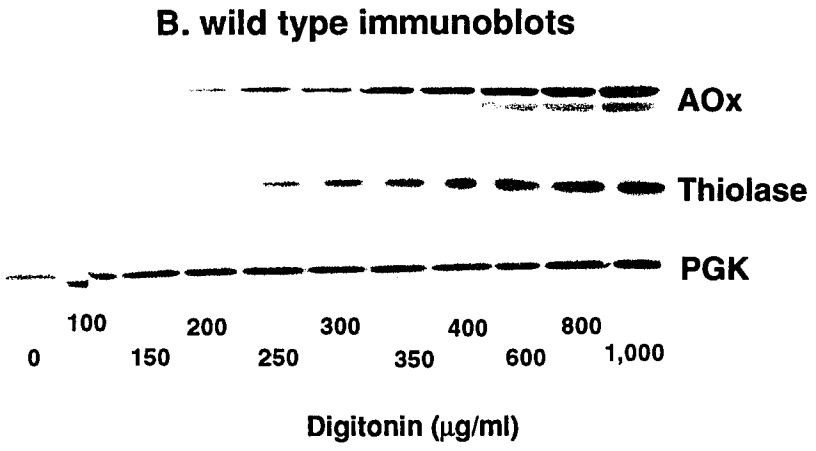
I tested, on wild type *S. cerevisiae*, the feasibility of selectively permeabilizing plasma and intracellular membranes with digitonin. Wild type yeast were grown in glycerol plus oleic acid to induce peroxisomes and were converted to spheroplasts as described in Materials and Methods. The spheroplasts were treated with different concentrations of digitonin as indicated in Fig. 3-2. Two cytosolic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, which was measured enzymatically) and phosphoglycerate kinase (PGK, which was quantitated by immunoblotting) were released from cells in parallel (Fig. 3-2, dashed lines). Substantial amounts of both enzymes appeared in the supernatant at the lowest concentration of digitonin tested, 100 μg/ml. Release was approximately 70% complete at 200 μg/ml. With the exception of one data point (at 600 μg/ml), the data for the two cytosolic enzymes agreed closely, as they should for two enzymes that are being released from the same intracellular compartment.

Fumarase, a marker enzyme for the mitochondrial matrix, remained in the spheroplasts at the low concentrations of digitonin

Figure 3-2. Release of proteins from wild type yeast spheroplasts by digitonin titration. Aliquots of spheroplasts were incubated with digitonin at the concentrations indicated for 20 min at 4°C and then were centrifuged to pellet the cells. Peroxisomal enzymes: catalase, thiolase and AOx. Mitochondrial enzyme: fumarase. Cytosolic enzymes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PGK. The activities of GAPDH, catalase and fumarase in the supernatants were assayed as described in Materials and Methods. The amounts of PGK, thiolase and AOx protein in the supernatants were determined by immunoblotting as shown in Fig. 3-1. (*A*) Plot of the amount of each enzyme released, normalized to the amount found in the supernatant at 1000 μg/ml of digitonin. (*B*) Raw immunoblot data. Each antiserum was applied to a separate nitrocellulose blot. The relevant section of each film is shown as a computerized image.

Digitonin cell fractionation: wild type yeast





that released cytosolic enzymes. Fumarase was released at approximately 600 µg/ml. This difference in the concentrations of digitonin that were required to permeabilize the plasma membrane and the mitochondrial membranes was reproducible and sufficient to clearly distinguish experimentally between enzymes in the cytosol and enzymes in the mitochondria.

Catalase A was used as a marker enzyme for peroxisomes. It is the only active catalase isozyme in the strains used here (Cohen et al., 1985) (Chapter 2). Catalase A was released to the supernatant at concentrations of digitonin similar to those required for mitochondrial enzymes (Fig. 3-2). Two other peroxisomal enzymes, thiolase and acyl-CoA oxidase (AOx), emerged together with catalase, as would be expected if their release depended on permeabilization of the same membrane. Each of the three peroxisomal enzymes began to emerge slightly before fumarase (Fig. 3-2), and this small difference appeared consistently in other experiments. Control experiments with total cell homogenates prepared with glass beads demonstrated that release of all enzymes was essentially complete at 1 mg/ml of digitonin (data not shown).

These data indicate that digitonin may be applied to the selective permeabilization of *S. cerevisiae* membranes.

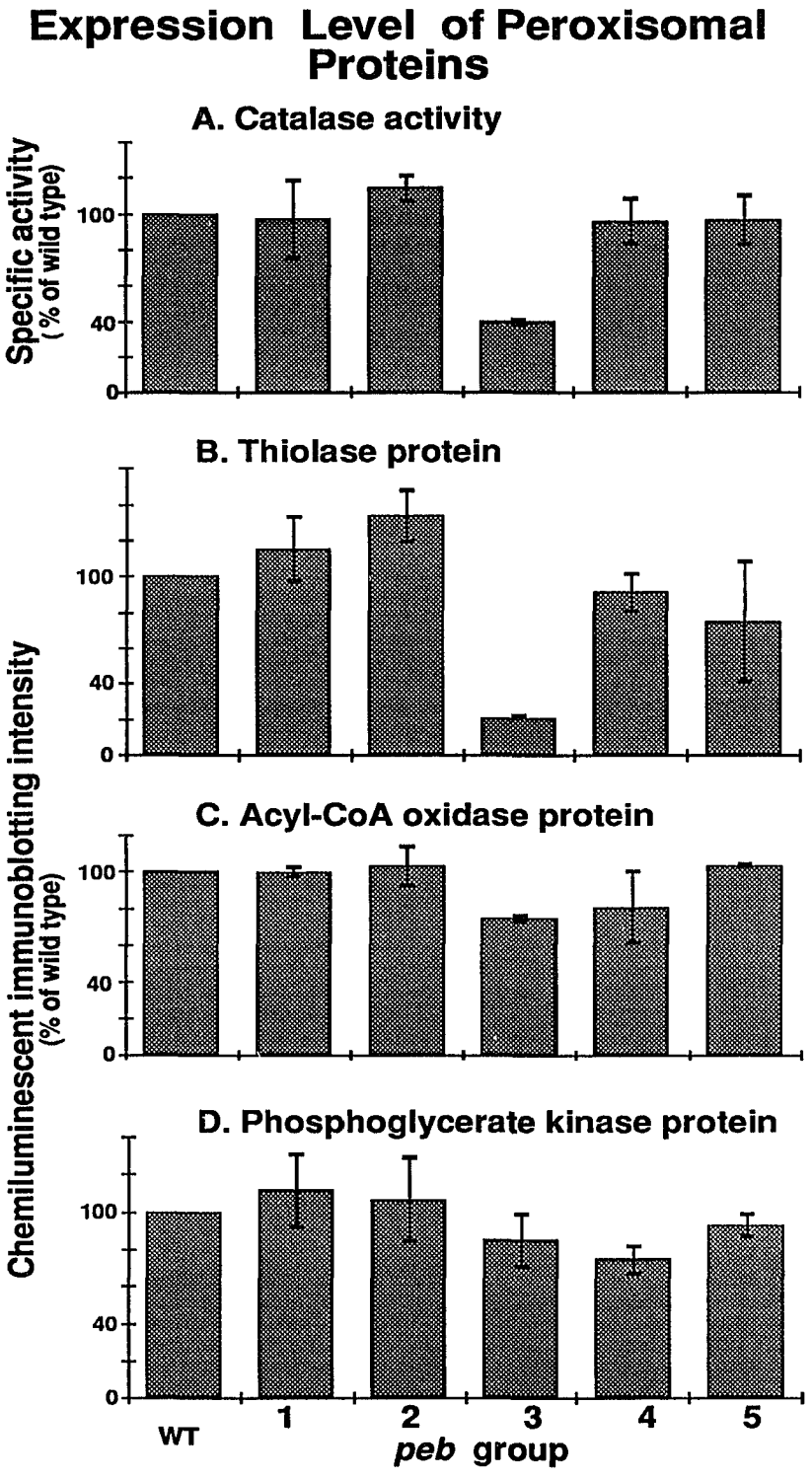
Peroxisomal Protein Expression in the Peb Mutants

The abundance of peroxisomal proteins was quantified in the mutant cells (Fig 3-3). There was no significant change in the amount of catalase, thiolase or acyl-CoA oxidase in mutants 1, 2, 4 or 5. Significantly less catalase activity and thiolase protein were found in *peb3*. Acyl-CoA oxidase protein was present in *peb3* at levels close to those in wild type cells.

Application of the Digitonin Permeabilization Method to Peroxisome Biogenesis Mutants of S. cerevisiae.

The subcellular locations of the three peroxisomal proteins in our five peroxisome biogenesis mutants were analyzed with the digitonin permeabilization technique. The yeast were grown in

Figure 3-3. Expression levels of peroxisomal proteins in wild type and mutant yeast cells. Three separate experiments were carried out and the results are expressed as the means; the error bars indicate the standard deviations. Total cell homogenates were prepared with glass beads as described in Methods. Catalase was measured enzymatically. This activity belongs to the peroxisomal isozyme, catalase A. The gene for catalase T, a cytosolic isozyme in *S. cerevisiae*, is nonfunctional in these cells (Cohen et al., 1985). Thiolase, acyl-CoA oxidase and phosphoglycerate kinase were determined by quantitative immunoblotting and densitometry. Equal amounts of homogenate protein from wild type and each mutant were subjected to SDS polyacrylamide gel electrophoresis. The amount of each protein found in the mutants is represented as the percentage of that in wild type cells. In the case of acyl-CoA oxidase, the error bars represent the range in two experiments.


Expression Level of Peroxisomal Proteins
A. Catalase activity
Specific activity (% of wild type)
100
40
0
B. Thiolase protein
100
40
0
C. Acyl-CoA oxidase protein
Chemiluminescent immunoblotting intensity (% of wild type)
100
40
0
D. Phosphoglycerate kinase protein
100
40
0
WT
1
2
3
4
5
peb group

medium containing glycerol (so that peroxisome function was not required) and oleic acid (to induce peroxisomes if possible). The digitonin concentrations needed to release cytosolic proteins and to release mitochondrial protein from the mutant spheroplasts (Figs. 4-6) were generally similar to those required with wild type cells (Fig. 3-2). Exceptionally, fumarase emerged at somewhat lower digitonin concentrations in *peb*1 (Fig. 3-5A). Thus, as expected, the *peb* mutations do not affect the cellular topology of cytosolic or mitochondrial proteins, and, at least in *peb*2, 3, 4, and 5, do not affect the susceptibility of the mitochondrial and plasma membranes to digitonin. The change in *peb*1 needs to be further investigated in a null mutant.

Distribution of Peroxisomal Enzymes in Peb Mutants in Which Peroxisomes Are Not Recognizable Morphologically.

Peroxisomes were not found in mutants *peb2*-1 and *peb4-1* despite extensive morphological searching (Chapter 2). Digitonin treatment of these two mutants caused the release of catalase, thiolase and acyl-CoA oxidase together with the cytosolic marker enzyme, PGK (Fig. 3-4). These data demonstrate that the three peroxisomal

enzymes are mislocated to the cytosolic compartment in these cells. This is consistent with the morphological absence of peroxisomes, and with the known facts of peroxisome biogenesis. Peroxisomal proteins are synthesized on free polyribosomes from which they are released into the cytosol when synthesis is complete (Lazarow and Fujiki, 1985); without peroxisomes, they remain in the cytosol.

Distribution of Peroxisomal Enzymes in Peb Mutants in Which Peroxisomes Are Present.

***peb1-1 mutant*:** Thiolase and catalase were released separately from *peb1-1* spheroplasts by digitonin (Fig. 3-5A, compare open circles and squares). Thiolase emerged with the cytosolic marker, PGK. Catalase came out at digitonin concentrations slightly lower than those that release fumarase. These data demonstrate that catalase is packaged within peroxisomes, but thiolase is not.

Acyl-CoA oxidase was released at approximately the same digitonin concentrations as catalase, consistent with a peroxisomal

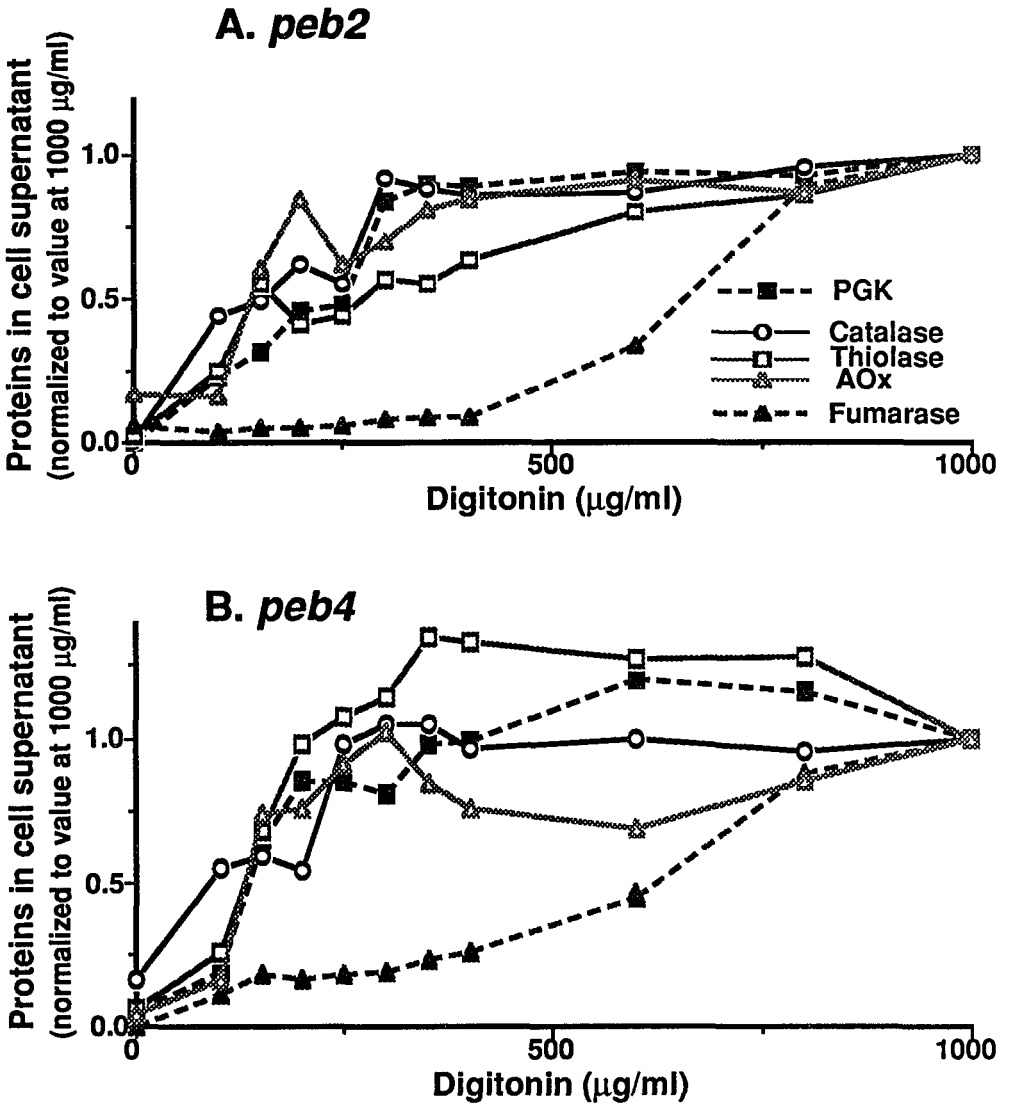
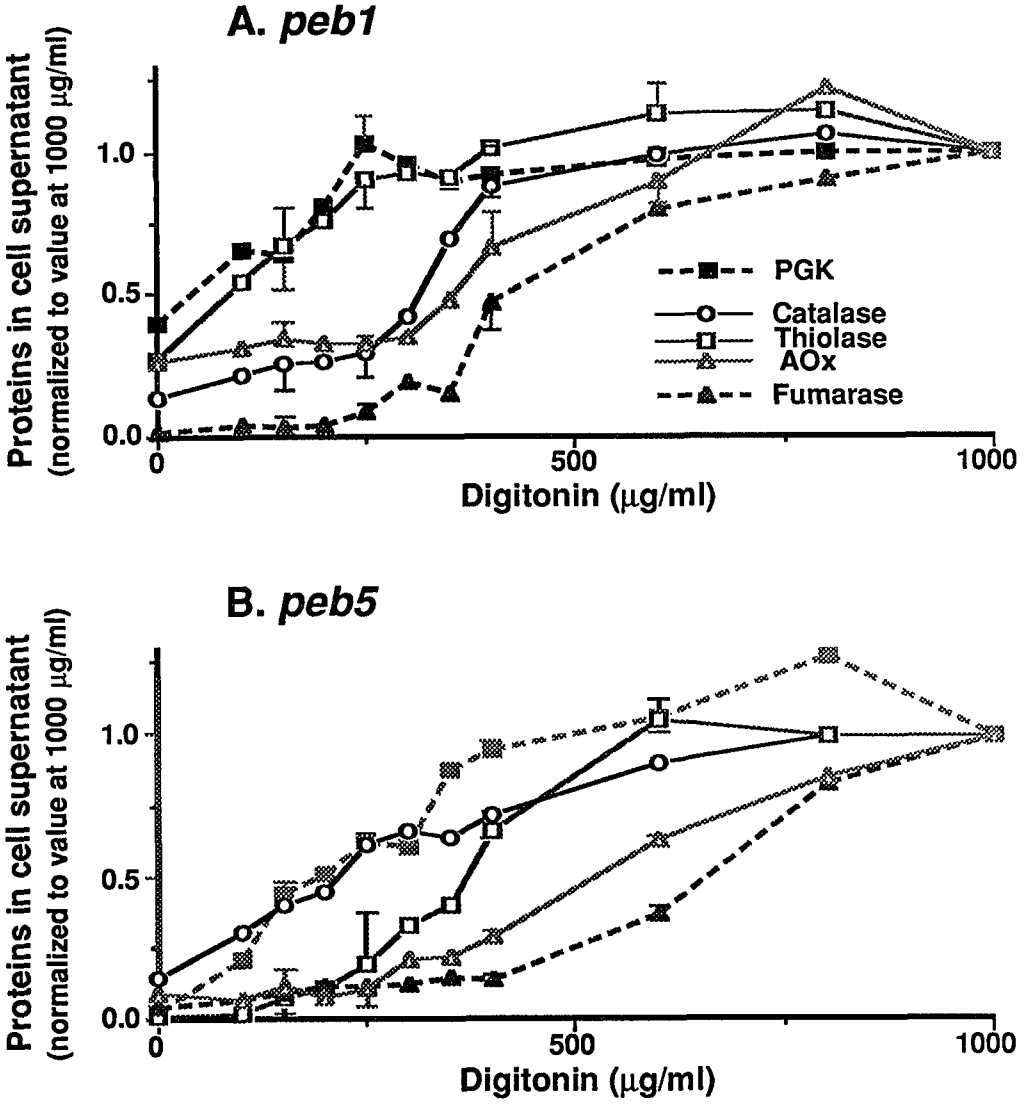


Figure 3-4. Digitonin titration: mutants with no recognizable peroxisomes. (A) peb2-1. (B) peb4-1. Symbols are the same as in Fig. 3-2.

Figure 3-5. Digitonin titration. Mutants that contain peroxisomes. (*A*) *peb1-1*. (B) *peb5-1*. Each plot represents the mean values of two entirely separate experiments. The range of the individual values is indicated for digitonin concentrations of 150, 250, 400 and 600 µg/ml. For the sake of clarity, only the upper or lower half of an error bar is shown. Where error bars are not visible at these digitonin concentrations, they lie within the symbol. Symbols are the same as in Fig. 3-2.

Digitonin cell fractionation: mutants with peroxisomes



localization. A portion of both of these enzymes was found within the supernatants at the low digitonin concentrations where cytosolic proteins emerge, raising the possibility that packaging may be incomplete, leaving about 20-30% of these peroxisomal proteins in the cytosol.

The data shown represent the means of two entirely separate experiments. The modest sizes of the error bars, which indicate the range of values in individual experiments, demonstrate the reproducibility of the digitonin permeabilization technique and the consistent behavior of the mutant.

peb5-1 mutant: Catalase cofractionated with the cytosolic proteins in this mutant (Fig. 3-5*B*, open circles). Acyl-CoA oxidase behaved exactly as in wild type cells, emerging just before the mitochondrial marker (open triangles). Unexpectedly, thiolase was largely released between 250 and 400 μg/ml of digitonin. This is a greater digitonin concentration than is required for cytosolic proteins but lower than for acyl-CoA oxidase. The error bars demonstrate the reproducibility of these results in two experiments. Thiolase was unambiguously found in peroxisomes in this mutant by

immunogold labeling (Chapter 2). The significance of the release of thiolase at an intermediate digitonin concentration will be discussed below.

Selective packaging defects. These fractionation data demonstrate that mutants *peb1-1* and *peb5-1* contain some but not all of the peroxisomal proteins within the peroxisomes. Thiolase is left in the cytosol in *peb1* whereas catalase is left in the cytosol in *peb5*. Both of these proteins contain the correct targeting information to be packaged into peroxisomes. Catalase is expressed from a wild type gene that was put into the mutants by backcrossing, and this catalase is correctly imported into peroxisomes in two of the mutants. Thiolase of *peb1* is imported into peroxisomes in a diploid cell formed by mating *peb1* with a yeast strain lacking thiolase (Chapter 2). Therefore, these mutations must affect the packaging mechanism, not the targeting information.

Acyl-CoA oxidase appears to be assembled normally into peroxisomes in both selective packaging mutants. These data suggest that the import of acyl-CoA oxidase involves a mechanism

that differs from the one used by catalase, and differs from the one used by thiolase.

Distribution of Peroxisomal Enzymes in a Peb Mutant with Lower Expression of Some Peroxisomal Proteins.

The steady state expression levels of catalase and thiolase, were lower in *peb3-1* than in wild type cells, whereas the expression of acyl-CoA oxidase was approximately normal (Fig. 3-3). All three peroxisomal proteins were released together at digitonin concentrations between 200-400 μg/ml (Fig. 3-6). This is more digitonin than is needed for cytosolic proteins but less than for the mitochondrial proteins. It is also less digitonin than is needed for release of peroxisomal proteins in wild type cells. The intermediate amount of digitonin required to solubilize all three peroxisomal proteins in *peb3-1* was similar to the amount required to release thiolase in *peb5-1*. These and other data suggest that the properties of the peroxisome membrane may have been altered (see Discussion).

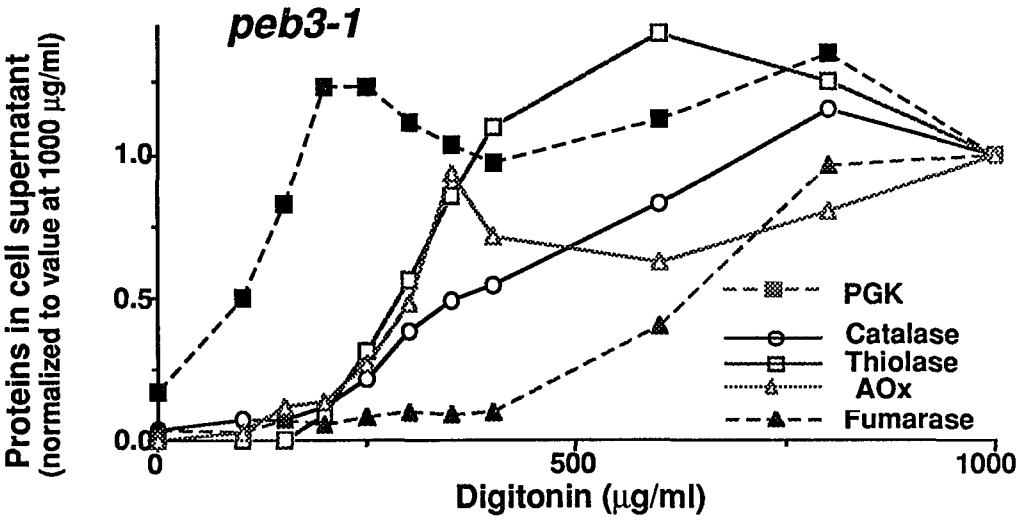


Figure 3-6. Release of proteins from mutant peb3-1, which has a low expression level of some peroxisomal proteins, by digitonin titration.

Discussion

Implications for Peroxisome Biogenesis

Mutant *peb3-1* has two distinct abnormalities in its peroxisomal phenotype. Two peroxisomal proteins (at least) were not induced normally by growth on oleate. One of these proteins is thiolase, which explains the faint thiolase immunofluorescence in the peroxisomes that was observed previously (Chapter 2). All three peroxisomal enzymes that were tested demonstrated the same dependence on digitonin concentration for their release from the spheroplasts, which is consistent with their location within peroxisomes. The peroxisomal localization was confirmed for thiolase in *peb3-1* by immunoelectron microscopy (Chapter 2). Unexpectedly, the amount of digitonin that was required to solubilize the three peroxisomal enzymes was distinctly less than the amount required to solubilize the mitochondrial marker enzyme, in contrast to wild type cells (Fig. 3-6). This suggests that the properties of the peroxisomal membrane, perhaps its ergosterol content, or perhaps some other characteristic, may be altered in *peb3-1*. One may speculate that this alteration in membrane

properties could be the result of the lack of induction of some other peroxisomal protein(s). This will be explored further in the future on a null mutant.

All peroxisomal proteins tested in mutants *peb2*-1 and *peb4-1* were found in the yeast cytosol (Fig. 3-4). This confirms and extends our previous immunofluorescence, cytochemical and immunolocalization experiments, in which peroxisomes were not seen (Chapter 2). It is consistent with the fact that peroxisomal proteins are imported posttranslationally into preexisting peroxisomes from the cytosol (Lazarow and Fujiki, 1985). No mislocalization of peroxisomal proteins to other organelles was detected.

Digitonin titration of mutants *peb1-1* and *peb5-1* demonstrated the mislocalization to the cytosol of individual peroxisomal proteins (Fig. 3-5). In the case of *peb1-1*, thiolase fractionated with the cytosolic marker enzymes, whereas the other two peroxisomal enzymes emerged just before the mitochondrial marker, as in wild type cells. These biochemical results agree with our morphological data.

Each of the three peroxisomal enzymes was released from *peb5-1* spheroplasts in a distinct fashion (Fig. 3-5*B*). Catalase emerged with the cytosolic proteins, consistent with the previous observation that there was no catalase cytochemical reaction product in peroxisomes (Chapter 2). Acyl-CoA oxidase responded to digitonin as it did in wild type cells, emerging just before the mitochondrial marker enzyme. Thiolase release, unexpectedly, was intermediate between the two other peroxisomal enzymes, and distinctly different from both. Could thiolase be mislocalized to some other organelle? No: it was found within peroxisomes by immunogold labeling (Chapter 2). Could acyl-CoA oxidase be mislocalized to some other organelle? This appears unlikely in view of the fact that (i) the release of the oxidase from *peb5-1* appears to be indistinguishable from its wild type behavior and (ii) when acyl-CoA oxidase is not packaged into peroxisomes in other mutants, it is found in the cytosol.

If thiolase and acyl-CoA oxidase are both located in peroxisomes in *peb5-1*, why do they emerge at different concentrations of digitonin? One possibility is that there is some

difference in the forces retaining them within the organelle as digitonin begins to permeabilize the peroxisomal membrane. In the case of rat liver, the oxidase remains partially associated with peroxisomes that have been damaged by freezing and thawing or by low concentrations of Triton X-100, whereas catalase and thiolase are easily released from damaged peroxisomes. This is thought to be the result of the binding of the oxidase to other proteins within the peroxisomal matrix. The oxidase is released from rat liver peroxisomes by higher concentrations of Triton X-100 (Alexson et al., 1985). A similar mechanism might explain the data of *peb5-1*. A protein with which thiolase normally associates might be missing from peroxisomes in the *peb5* mutant, so that thiolase leaks out more easily. Whatever the explanation, it must account for the fact that no differential leakage of peroxisome enzymes was observed from wild type spheroplasts or from *peb3-1* spheroplasts. All three peroxisomal enzymes emerged together from these strains. Further experiments are required in the future to investigate this question.

It is noteworthy that the data on the mutants with selective packaging defects suggest that there are at least three distinct

pathways, or three branches in a pathway, for the import of proteins into peroxisomes (Fig. 3-7). Among catalase, thiolase and acyl-CoA oxidase, the import of either of the first two could be abolished without affecting the import of the others. One possibility is that these three proteins may have different topogenic features and use different receptors. *S. cerevisiae* thiolase may use an amino-terminal targeting sequence like that of rat liver thiolase (Osumi et al., 1991; Swinkels et al., 1991) . Catalase has a carboxyl-terminal SSNSKF (a variant of SKL) that is sufficient for import in *S. cerevisiae in* addition to an internal signal (Kragler et al., 1993). The topogenesis of *S. cerevisiae* acyl-CoA oxidase is unknown. It lacks an SKL-like carboxyl-terminus and its amino-terminal sequence does not appear to resemble of that of thiolase (Dmochowska et al., 1990). Perhaps it contains internal targeting peptides like acyl-CoA oxidase from *C. tropicalis* (Small et al., 1988). Thus, one interpretation of our data is that there may be three distinct receptors that recognize peroxisomal targeting features, one for

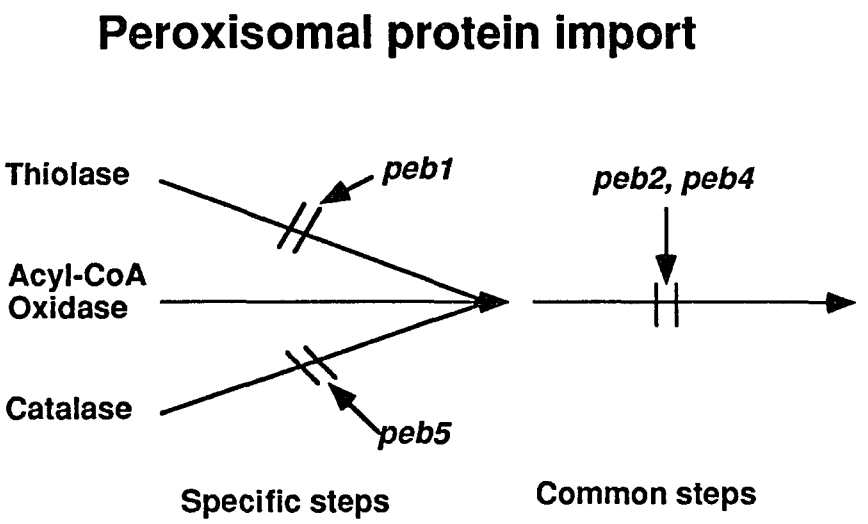


Figure 3-7. Speculative model of peroxisome biogenesis, suggested by the observed packaging defects. Peb2 and peb4 prevent the packaging of all three enzymes whereas peb1 and peb5 each affect just one. One possibility is that the PEB1 and PEB5 genes encodes receptors and the PEB2 and PEB4 genes encode parts of the translocation machinery. The temporal and topological relationships of these gene products are unknown.

each of the three peroxisomal proteins tested. Whether the observed selective packaging defects are due to mutations in these putative receptors, or to other defects, remains to be elucidated by further experiments.

New Techniques

The application of increasing concentrations of digitonin proved to be an effective method to selectively permeabilize the membranes of *S. cerevisiae*. Two cytosolic enzymes were released together at low digitonin concentrations. Three peroxisomal enzymes were released together from wild type peroxisomes at substantially higher digitonin concentrations. Fumarase was released from mitochondria at an even higher digitonin concentration (Fig. 3-2). I assume that digitonin probably functions by forming complexes with ergosterol in the yeast membrane, although that has not been demonstrated in these experiments. Regardless of mechanism, the digitonin titration of yeast spheroplasts permits one to determine the intracellular location of enzymes without the mechanical disruption associated with conventional cell fractionation methods.

There are a couple of factors which can affect the release

pattern of proteins by this method. Different strains have different sensitivity to Zymolyase digestion which could affect the degree of spheroplast conversion and then the release pattern of proteins upon digitonin titration. The commercially available digitonin is not a pure chemical. Its purity can vary from batch to batch. This variation will change the release pattern of proteins. However, the intracellular location of a certain protein can be determined in a single experiment by comparing its release pattern upon digitonin titration with the release patterns of proteins from known subcellular compartments. The release pattern of proteins upon digitonin titration is quite reproducible in the same strain and using the same batch of digitonin, as shown in Fig. 3-5.

Moreover, because digitonin gently permeabilizes the plasma membrane without affecting the integrity of organelles, it could be used for *in vitro* studies of protein import into organelles in yeast. Digitonin permeabilization of mammalian cells has been used successfully for *in vitro* import assays of nuclear proteins (Adam et al., 1990) and for studies of endosome acidification and function (Diaz and Stahl, 1989).

Another technological advance in this study was that conditions were found for the linear assay of proteins on western blots, exploiting the sensitivity of the chemiluminescence detection procedure (Fig. 3-1). The application of chemiluminescence detection to immunoblotting has greatly increased the sensitivity of antigen measurements. However, to the best of our knowledge, this is the first instance in which the quantitative aspects of this procedure have been systematically tested. The observed linearity permits quantitative determination of antigens.

Chapter 4

The cloning of PEB1 gene and characterization of the gene product in *Saccharomyces cerevisiae*

Introduction

In Chapters 2 and 3, I have described the isolation and characterization of new *peb* mutants in *S. cerevisiae* which fall into five complementation groups. Two groups do not have morphologically recognizable peroxisomes. All three peroxisomal proteins examined (thiolase, catalase and acyl-CoA oxidase) mislocated into the cytosol in them. In one complementation group, *peb3*, the proliferation of peroxisomes and expression of some peroxisomal proteins is decreased in oleate containing medium. In another two complementation groups, normal looking peroxisomes are present, but they are selectively deficient in the import of one

subset or another of peroxisomal proteins. *Peb1* does not package thiolase, but the import of catalase and AOx is normal; *Peb5* does not package catalase, but the thiolase and AOx are in peroxisomes.

In this study, I took this project one step further by cloning the PEB1 gene and characterizing the gene product. I demonstrated that the PEB1 encodes a 42.32 kDa hydrophilic protein which is essential for the import of thiolase. This protein is likely located inside the peroxisome. The possible role of Peb1p in peroxisome biogenesis is discussed.

Materials and methods

Yeast strains, plasmids and culture condition

Yeast strains used in this study are listed in Table 4-1. The plasmids used in this study are listed in Table 4-2. The maps of 5 of them are shown on Figure 4-1. Peroxisome induction was as described in Chapter 3 except for strains JW88 and JW72. In these two strains, the cells were preculured twice in minimal medium plus any essential nutrients instead of YPD, for the prevention of plasmid loss.

Isolation of PEB1 gene

The mutant m6-D1 was transformed with a Ycp50 based genomic library constructed by Rose et al. (1987) which contains a URA3 marker. About 1 x 10^9 cells were spread on SC (synthetic complete)-uracil plates. The Ura+ transformants were screened for restored peroxisomal function by their ability to grow on YNO plates. About 8-10 transformants were first pooled and resuspended in 100 μl

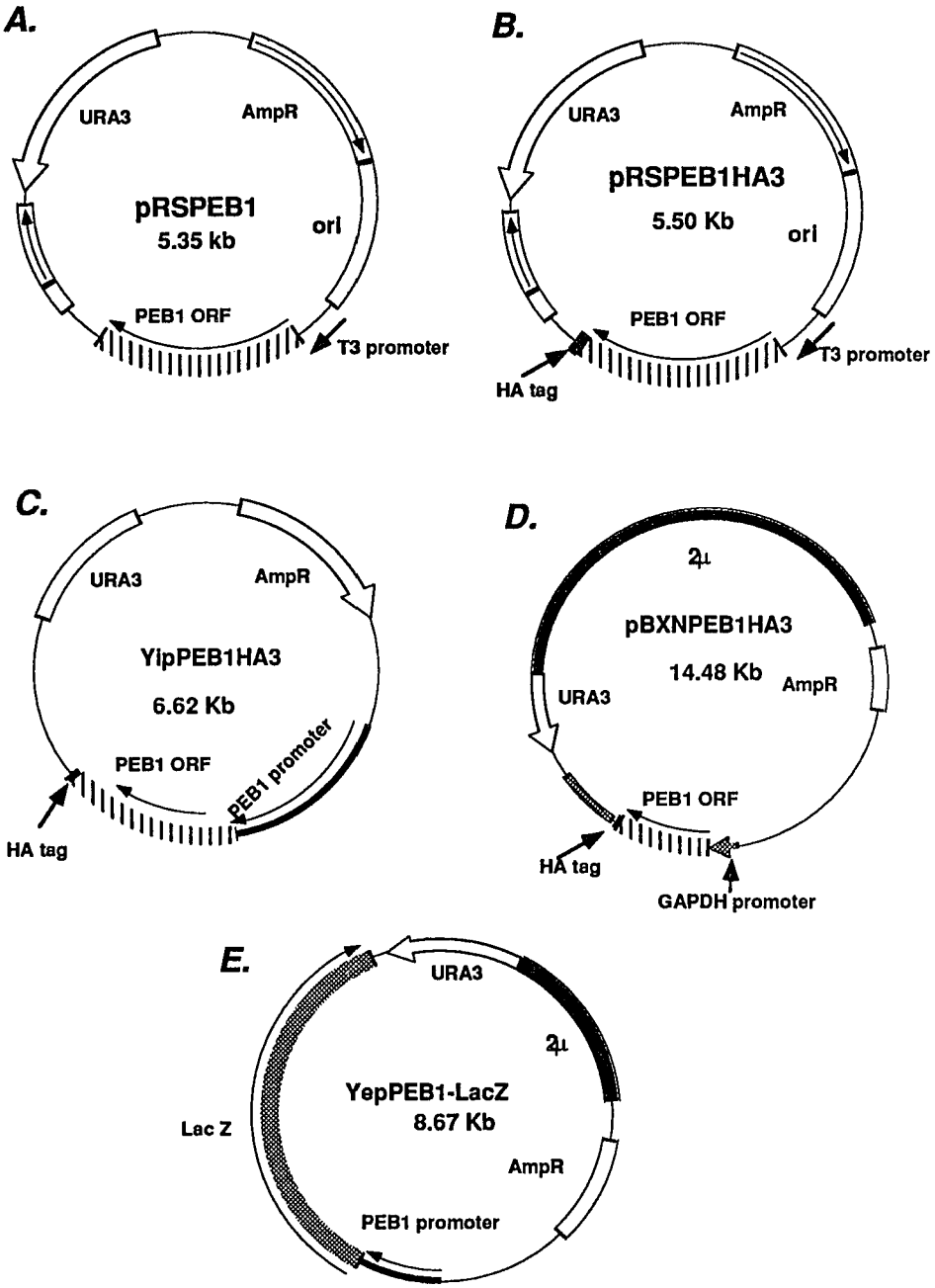
Table 4-1 Yeast Strains used in Chapter IV

Name	Genotype	Sources
JW68-3A	*Matα, ura3-1, trp1-1, arg4, ctt1-1*	Chapter 2
m6-D1	*Mata, peb1-1, ura3-1, trp1-1, ctt1-1*	Chapter 2
JW72	*Matα, ura3-1, trp1-1, arg4, ctt1-1* [YepPEB1-LacZ]	This study
JW75	*Matα, ura3-1, trp1-1, arg4, ctt1-1, peb1::TRP1*	This study
JW86	*Matα, ura3-1, trp1-1, arg4, ctt1-1, peb1::TRP1* [YipPEB1HA3]	This study
JW88	*Matα, ura3-1, trp1-1, arg4, ctt1-1, peb1::TRP1* [pBXNPEB1HA3]	This study

Table 4-2 Plasmids

Name	characteristics	Source
YcpM6R	10 kb fragment complementing the *peb1* mutant. Came from the genomic library (Rose et al., 1987). URA3. Ycp50 based	This study
pSM1	3 kB fragment complementing *peb1* mutants. Cloned in pRS314 (see below)	This study
pRSPEB1	PEB1 ORF cloned in pRS314. T3 promoter. See Fig. 4-1*A*.	This study
pRSPEB1HA3	PEB1 ORF tagged with 3 copies of the HA epitope cloned in pRS314. T3 promoter. See Fig. 4-1*B*.	This study
YipPEB1HA3	Triple-tagged PEB1 including 1124 bp upstream untranslated sequence cloned in pRS304. See Fig. 4-1*C*.	This study
pBXNPEB1HA3	Triple-tagged PEB1 ORF cloned in pAB23BXN. GAPDH promoter. 2 μ. See Fig. 4-1*D*.	This study
pJW17	peb1::TRP1. The Bgl2 fragment of the PEB1 gene was replaced with the TRP1 gene. Based on pSM1. See Fig. 4-6*A*.	This study
YepPEB1-LacZ	750 bp of 5'upstream untranslated region of the PEB1 gene fused in frame with LacZ gene in Yep368. See Fig. 4-1*E*.	This study
pJW42	the PEB1 gene including 1124 bp upstream untranslated sequence cloned in pRS304.	This study
pAB309Δ	TCM1 gene cloned in pGEM1.	(Schultz & Fressen, 1983)
pRS314	TRP1. CEN4	(Sikorski & Hieter, 1989)
pRS304	TRP1. Yip.	(Sikorski & Hieter, 1989)
pRS306	URA3. Yip	(Sikorski & Hieter, 1989)
pAB23BXN	URA3. GAPDH promoter. 2μ	(Schild et al., 1990)
Yep368	URA3. LacZ. 2μ	(Myers et al., 1986)

Figure 4-1 Plasmid maps. See Table 4-2 for description. Each map is drawn to scale. The total size of the plasmid is indicated. The PEB1 ORF in (A), (B), and (D) contains 49 bp of 5′ UTR (cloned from the Bgl2 site upstream of the ATG). The PEB1 ORF in (A) contains 294 bp of 3′ UTR.


A.
URA3
AmpR
pRSPEB1
5.35 kb
ori
PEB1 ORF
T3 promoter
B.
URA3
AmpR
pRSPEB1HA3
5.50 Kb
ori
PEB1 ORF
T3 promoter
HA tag
C.
URA3
AmpR
YipPEB1HA3
6.62 Kb
PEB1 ORF
PEB1 promoter
HA tag
D.
2μ
pBXNPEB1HA3
14.48 Kb
AmpR
URA3
PEB1 ORF
HA tag
GAPDH promoter
E.
URA3
2μ
YepPEB1-LacZ
8.67 Kb
Lac Z
AmpR
PEB1 promoter

sterile water in a well of 96-well plates. These were then transferred to YNO plates using the multi-prong inoculator (frogging technique) for growth. The plasmid with the ability to complement the *peb1* mutation was isolated from the yeast cells according to Hoffman and Winston (1987) and transformed into *E. Coli* by electroporation (Dower et al., 1988).

DNA sequencing

The plasmid pSM1 (Table 4-2), which contains a 3.0 kb fragment capable of complementing the *peb1* mutation, was subjected to nested deletion using Exonuclease III (Henikoff, 1984). The nested subclones were sequenced using T3 primer. The few gaps of this strand not sequenced and the opposite strand were sequenced using synthesized oligonucleotides. All the sequences were determined using dideoxy (Sanger) sequencing methods (Sanger et al., 1977).

In vitro transcription and translation

The reagents for in vitro expression of proteins were from Promega (Madison, Wisconsin). The protocols were according to the

manufacture. The plasmids pRSPEB1 and pRSPEB1HA3 were transcribed with T3 RNA polymerase after linearization. The RNA was extracted with phenol/chloroform, ethanol-precipitated, and translated in vitro with reticulocyte lysate and labeled with ^{35}S trans-label (NEN, Boston, Massachusetts). A portion of the translation products was analyzed with SDS-PAGE and detected by fluorgraphy.

Epitope tagging

The 9 amino acid residue hemagglutinin epitope (YPYDVPDYA) (Wilson et al., 1984), which can be recognized by the monoclonal antibody 12CA5 and is referred to as the HA epitope in the rest of this thesis, was used for tagging. Three tandem copies of HA epitope were added to the C-terminus of the PEB1 gene by 2 consecutive PCR reactions.

The first PCR reaction added one HA epitope to the extreme C-terminus of the PEB1 gene. The internal primer sequence (5'GCT GCT GGT TCA AAC TTT G 3') corresponds to the nucleotide 1201 to 1219 in the

PEB1 gene and has been synthesized for the purpose of sequence determination. The primer used to add the HA epitope is 5'GTG **TCA** AGC GTA GTC TGG GAC GTC GTA TGG GTA ACC TAA GCC GTT CCA TAC AAA TA 3'. It is complementary to the plus strand of the PEB1 gene. The underlined sequence of the primer is from the PEB1 gene. The sequence for the HA epitope was inserted between the codon encoding the last amino acid residue and the stop codon (**TCA** in bold type) of the PEB1 gene. The wild type PEB1 gene was used as template. The PCR product obtained with these two primer was digested with BamH1 (see Fig. 4-3D). The downstream portion of the digestion product was used to replace the corresponding part of the wild type PEB1 gene. The region in the tagged gene introduced by PCR was sequenced. The amino acid sequence of the C-terminus of the tagged Peb1p is: STAWDGNLFVWNGLGYPYDVPDYA*, with the sequence from Peb1p underlined. The star denotes the stop codon.

A similar strategy was used to introduce two more copies of the HA epitope to the Peb1p in the second PCR reaction (Tyers et al., 1992). This reaction used the tagged version of PEB1 gene as

template. The same internal primer used in the first PCR reaction was used in this reaction. The oligonucleotide for adding the second and third epitope was intended to be 5'TCA **TTA** GGC G(T)A ATC AGG AAC GTC ATA CGG GTA GGA TCC TGC ATA GTC CGG TAC ATC ATA GGG ATA GCC AGC GTA GTC TGG GAC GTC GTA 3', with the sequence encoding the HA epitope being underlined. The third-base in some codons encoding the same amino acid in these three epitopes was designed to be different to avoid the self-annealing in the PCR reactions (Tyers et al., 1992). The TTA (in bold type) was intended to be the stop codon. The intended amino acid sequence of the triple-tagged Peb1p was: STAWDGNLFV-WNGLG-YPYDVPDYAGYPYDVPDYAGSYPYDVPDYA*. The spacer between the first and second epitope was glycine (G), between the second and the third epitope was glycine and serine (GS). The PCR reaction was carried out and the product was digested with Bgl2. The downstream portion of it was used to replace the wild type copy of the PEB1 gene, generating the triple-tagged version. The part of the sequence introduced by PCR reaction in the triple-tagged gene was sequenced, which revealed that the 11th nucleotide T in the synthesized oligonucleotide for tagging, which was in parentheses,

was omitted during the synthesis. This error resulted in an incomplete third (and the last) epitope. It produced a frame shift at the eighth amino acid residue of this epitope which resulted in the addition of six extra amino acids and a new stop codon TGA. The resulting sequence of the minus strand is: **5'AG TCA** ***TAT GGA TTC ATT AGG CGA*** ATC AGG AAC GTC ATA CGG GTA GGA TCC TGC ATA GTC CGG TAC ATC ATA GGG ATA GCC AGC GTA GTC TGG GAC GTC GTA **3'**, with the sequence encoding the epitope underlined. The codons for the newly introduced amino acid residues due to the error are italized. The sequence from the downstream polylinker region is in bold type which include the stop codon ACT. The final sequence of the triple-tagged Peb1p is STAWDGNLFVWNGLGYPYDVPDYAGYPYDVPDYAGSYPYDVPDSPNESI*, with the epitope sequence being underlined.

Cell fractionation

Yeast cells were converted to spheroplasts according to the protocol described in Chapter 3. The homogenization of spheroplasts and differential centrifugation were carried out as described by Thieringer et al. (1991) The peroxisomes were isolated by a linear

Nycodenz density gradient centrifugation. The protocol was from Dr. G.M. Small (personal communication) which was based on Thieringer et al. (1991) and Lewin et al. (1990) with modifications. The gradient was prepared in a heat-sealable ultracentrifuge tube with 15 to 36 % (wt/vol) Nycodenz resting on a 2 ml cusion of 50 % (wt/vol) Nycodenz in 5 mM MES, pH 6.0, 1 mM EDTA, 0.24 M sucrose. The 25,000 g pellet fraction was resuspended in 5 mM MES, pH 6.0, 1 mM EDTA, 0.24 M sucrose and loaded on the gradient and centrifuged at 4° C in a Sorvall vertical rotor (Vt865B) at 36,000 rpm for 90 min. The fractions were collected for analysis.

Protease K treatment of organelles

This was based on Hohfeld et al. (1991) with minor modification. The 25,000 g pellet fraction from JW88, which consisted mainly of mitochondria and peroxisomes, was resuspended in 1.2 M sorbitol, 5 mM MES, pH 6.0 and divided into aliqots. The digestion was carried out in a 50 µl reaction with agarose bead coupled protease K (Sigma, P 9290) (25 mg/ml) in the absence and presence of 0.1% Triton X-100 at room temperature. The digestion was stopped at different

time points by adding 1 µl of 100 mM PMSF in ethanol.

Northern Blots

Yeast total RNA was prepared according to Cross and Tinkelenberg (1991) from cells grown to the middle log phase. They were separated on 4 % urea-polyacrylamide gel and transferred to Gene Screen Plus (NEN, Boston, Massachusetts) according to Stoeckle and Guan (1993). The DNA fragments were labeled with [α-^{32}P] dATP (NEN, Boston, Massachusetts) by random oligonucleotide-primed synthesis (Sambrook et al., 1989). The membrane was probed according to standard protocol (Sambrook et al., 1989).

Other methods

The DNA recombination techniques were generally from Sambrook et al. (1989). The yeast genetics were from Sherman et al. (1986). The one step gene disruption was according to Rothstein (1991). The carbonate extraction was according to Fujiki et al. (1982).

Materials

Nycodenz was purchased from accurate Chemical and Scientific Corporation (Westbury, N.Y.). The monoclonal antibody 12CA5 which recognizes the HA epitope was kindly provided by Dr Michael Shia (Boston University School of Medicine). The oligonucleotides were synthesized by Mount Sinai DNA Core Facility. Other reagents were from Premega, Sigma (St. Louis, MO) or New England Biolabs (Beverly, MA).

Results

Cloning of PEB1 by functional complementation

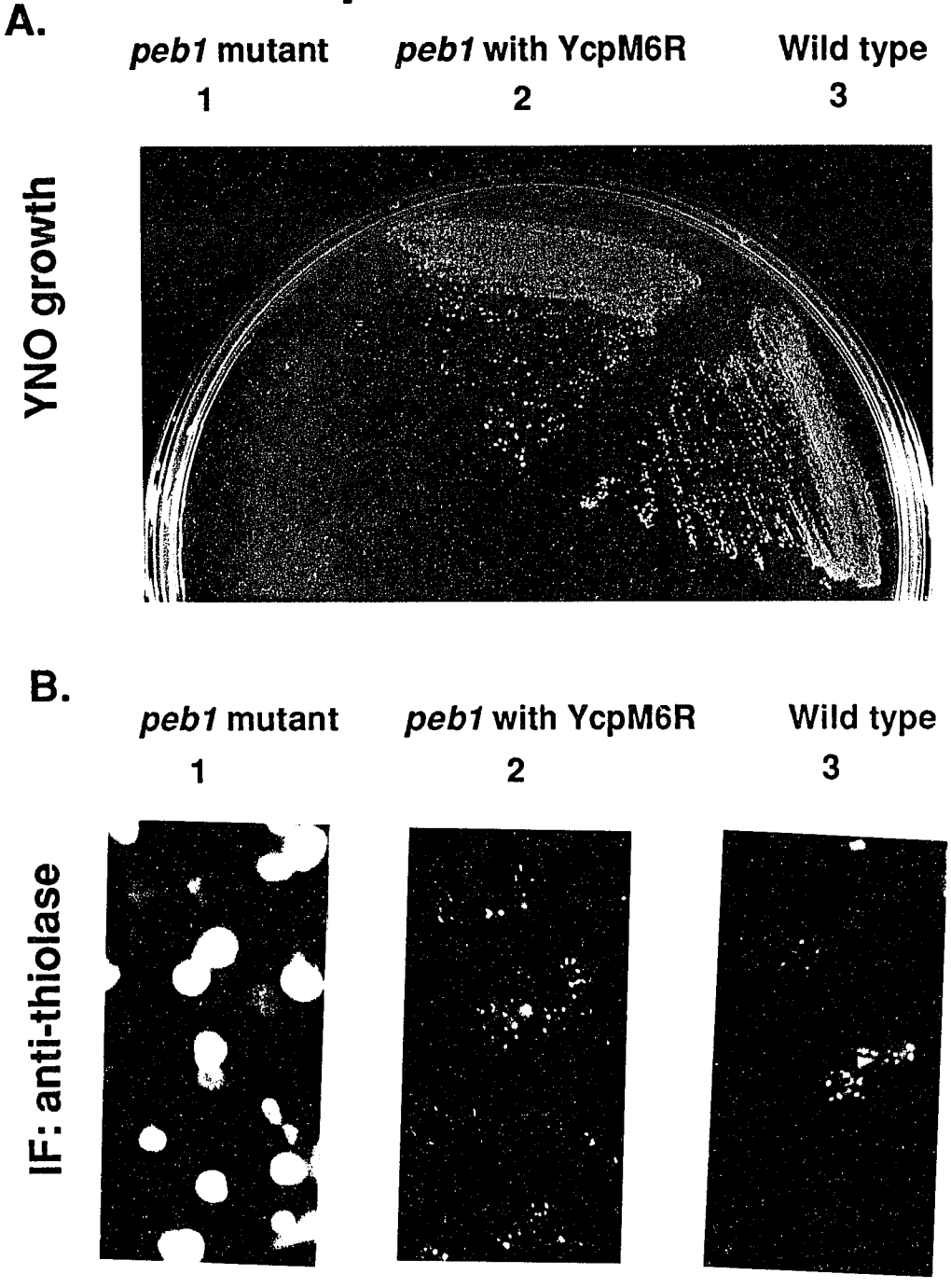
The mutant m6-D1 of the *peb1* group, which had been well characterized, was transformed with a yeast genomic library (Rose et al., 1987). About 3,000 transformants were obtained. Three of these showed positive growth on YNO medium and were then subcloned. To test the dependence of YNO growth on plasmids, these three clones were grown in YPD medium overnight, and then spread on YPD plates (about 200 cells/plate). After cells were grown into colonies of about 1 mm in diameter, they were transferred to SC-uracil plates with replica plating. Cells failing to grow on this medium, which indicate they had lost the plasmid, were tested for ability to grow on YNO plates (3 colonies from each clone were tested). By this test, all three colonies from one clone had lost the ability to grow on YNO plates after the plasmid was lost. This clone was termed m6-1R1 and was further analyzed. The other two clones were still Oleate+ after they lost the plasmid. They were then discarded.

Immunofluorescence analysis of M6-1R1 using anti-thiolase showed punctate staining, indicating the packaging of thiolase in peroxisomes (Fig. 4-2*B*). DNA was prepared (Hoffman and Winston, 1987) from the transformant for the retrieval of the plasmid. The initial attempts to transform the bacteria with the DNA by calcium chloride transformation were unsuccessful: no bacterial transformant was obtained in five consecutive experiments. Finally electroporation was used for bacterial transformation, and 5,000 transformants were obtained from 1 μl of yeast DNA solution. Plasmid DNAs were prepared from 3 independent transformants. Restriction mapping of them indicated they contained the same insert. When the plasmid was transformed back to the yeast mutant m6-D1, it restored the peroxisomal function of the mutant, as seen by growth on YNO medium and immunofluorescence with anti-thiolase (Fig. 4-2). This plasmid was termed YcpM6R.

The YcpM6R contains a 10 kb insert. The plasmid was digested

Figure 4-2 Functional complementation of the *peb1* mutant. (*A*) The growth on an oleate plate of m6-D1 (1), m6-D1 transformed with YcpM6R (2) and JW68-3A, wild type cells (3). *(B)* immunofluorescence of (1), (2), (3) with anti-thiolase.

Functional complementation of the *peb1* mutant



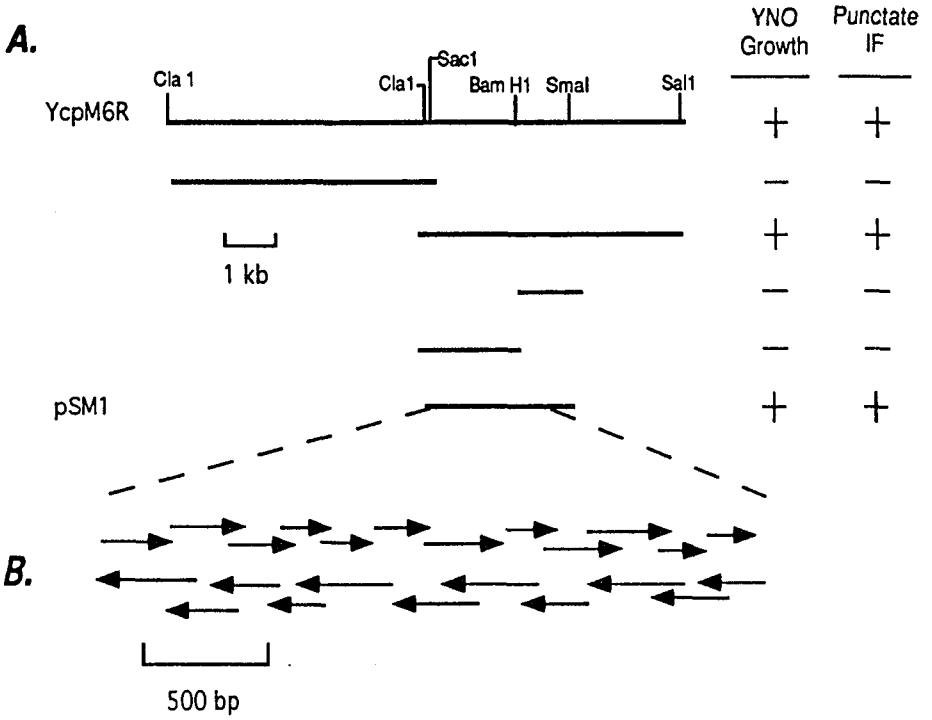
with different endonucleases (and combinations of endonucleases) and the restriction map of the insert was constructed. The defined restriction fragments of the insert (Fig. 4-3A were subcloned into pRS314. These subclones were transformed back into m6-D1 and were tested for their ability to complement the *peb1* mutation by growth on YNO plates and immunofluorescence with anti-thiolase. The smallest fragment with the ability to restore the growth on YNO plate of the mutant m6-D1 was a 3 kb SacI-SmaI fragment. This fragment also had the ability to restore the punctate type of fluorescence with anti-thiolase to the mutant (Fig. 4-3A). The plasmid containing this fragment was called pSM1.

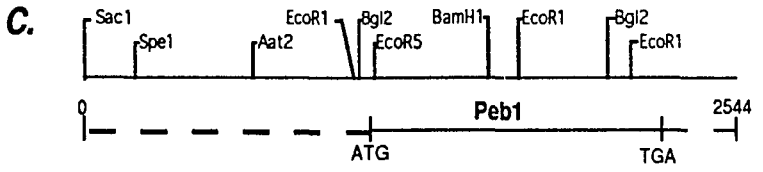
Sequence Analysis

The 2.5 kb region, as indicated in Fig. 4-3*A* and *B* of the 3 kb fragment in plasmid pSM1 was sequenced by the dideoxy-nucleotide chain-termination method (Sanger et al., 1977). Both strands were completely sequenced (Fig. 4-3B). As shown in figure 4-4, this region contained an open reading frame (ORF) of 1275 bp,

Figure 4-3 Localization of the PEB1 gene within the plasmid YcpM6R and sequence strategy. (*A*) The ability (+, -) to complement the mutant m6-D1 with fragments of the 10 kb genomic DNA insert in plasmid YcpM6R. Complementation was tested by growth on oleate plates (YNO growth) and immunofluorescence with anti-thiolase (Punctate IF). These two tests gave consistent results. (*B*) The direction and extent of sequence determinations are indicated by arrows. (*C*) A detailed restriction endonuclease map of the sequenced region and the identified ORF.

Localization of PEB1 gene and sequence strategy





corresponding to a putative protein of 375 amino acids with a molecular mass as 42.32 kDa. No intron was identified. The nucleotide at -3 position of the first ATG is A which is strongly conserved in yeast genes (Tuite, 1989). The C at +4 position is less common. In vitro transcription and translation of this ORF produces a band about 43 kDa (Fig. 4-5) consistent with the calculated molecular weight. These results indicated that the first ATG indeed is the translation start site.

Ruis's group (Filipits et al., 1993) has defined an oleate response region in the 5′ UTR of catalase A, with a consensus sequence as CGGNNNTNA in which N denotes any nucleotide. As this sequence has been found in the 5′ UTR of several genes encoding peroxisomal proteins, they called it "peroxisome box". It is found usually present in two boxes, with one direct and one inverted repeat. One such "peroxisome box" was found in the 5′ UTR of PEB1 gene (CGGCCTTCA, from -238 to -230 of the ATG) on the minus strand. Also on this strand downstream 7 nucleotide of this

Figure 4-4 Characterization of the PEB1 gene: Nucleotide and predicted amino acid sequence. The potential polyadenylation site is underlined. The potential "peroxisome box" sequences are underlined with dotted lines.

```
   1  GTGTCAACGGCCGAGCTCAA CTACTCAAATCCACCCTCTA ATTCTGGTGTTCGATAACAA   60
  61  TCAAAGTCGCGCTGGTTCCC ATCCCTTTGACTTTGTTCCA ATTCCACAGTCCAAATCCGA  120
 121  ATCCTTCCGCTTCTGATTCT TCGCAAGCCCATCAGCAGCG AACGGTCCAAACTCAAATAA  180
 181  CACTAGTAGTGACGCTACAG ACCCTCACCACAACAGACTA AGAGCCGTTTTGGATCACAT  240
 241  ATTCAACGTTGCTCAGAGGG GAACTTCTGATACCTCTGCA ACAACAGCACCCGGAGCACA  300
 301  AACTGTTCACAACCAAGGAC GTAATGACTCATCGTCCTCT GATACAACGCAAGGAAGTTC  360
 361  CTTTTTGGAAAATATTTCAC GATTAACAGGCCATTTTACG AATGGCTCAAGAGACAACAA  420
 421  TAACGACAATAACCATAGCA ATGATCAACAACGAGGTGGA AGTACTGGTGAGAACAACAG  480
 481  AAATAACTTGTTTTCCTCCG GTGTTGCCAGTTATAGAAAT CAAAATGGTGATGTTACTAC  540
 541  CGTCGAACTACGCAACAAMA ATTCTGCTGCCTTTCCTCCT ACAGACGAAAATCCCTCTCA  600
 601  AGGCCAAGGTTCAAGCAGTT CGGACACCACCATTCATAAC GACGTCCCTAATGATAACAA  660
 661  TGAGCAACGATCATCACAAT AAACAATACCGCTTCTGAAG TATGTTAATATGAAATATGT  720
 721  CCTATTGGCAGTCATCCATT TTCAGACATTATTTTCTACT CCTTTTACCTTTTTTTTATC  780
 781  TGCATCTTTATATATATATA TCATATACTTATTTTTCTTT ACTTCTACTTAAAGTTCTAT  840
 841  AATCATTGTTAATCAGAAAT TTGCATGTCTCCAGCAAGTA AATATGAAGGCCGAGAAATT  900
 901  AAACCACCGACATAACCTCC TCTGAAAAGTCGTTTCGCAA AACAGTAAAAGGTGAAAACA  960
 961  CTTTTAAGAACTGTGGAACA CAAACTGTGTTTTCTTACCA TAAAATCTTTTACCGGAGGT 1020
1021  CGAAATCGTTTTCTTTCTGA TTTGGAATTCGACGCTCGAA AAGTGCAGAGATCTCATATT 1080
1081  GTTAACGGACTATCATCTAA CTTTTTGCATAATTTATACA ACATGCTCAGATATCATATG 1140
   1                                                 M  L  R  Y  H  M    6
1141  CAAGGTTTTAGTGGGTACGG TGTCCAGTATTCTCCCTTTT TCGATAACAGGCTCGCGGTA 1200
   7   Q  G  F  S  G  Y  G   V  Q  Y  S  P  F  F   D  N  R  L  A  V   27
1201  GCTGCTGGTTCAAACTTTGG CCTGGTTGGGAATGGAAAAT TGTTCATCCTTGAGATTGAT 1260
  28   A  A  G  S  N  F  G   L  V  G  N  G  K  L   F  I  L  E  I  D   47
1261  CGTTCAGGTAGAATAGTGGA AGTTAATTCCTTTTTAACAC AGGATTGTTTATTTGATCTT 1320
  48   R  S  G  R  I  V  E   V  N  S  F  L  T  Q   D  C  L  F  D  L   67
1321  GCATGGAACGAAAGTCATGA AAACCAAGTGTTGGTTGCAC AGGGCGATGGTACATTACGC 1380
  68   A  W  N  E  S  H  E   N  Q  V  L  V  A  Q   G  D  G  T  L  R   97
1381  TTGTTTGATACAACCTTTAA AGAGTTTCCTATTGCTATAT TTAAAGAGCATGAACGAGAA 1440
  88   L  F  D  T  T  F  K   E  F  P  I  A  I  F   K  E  H  E  R  E  107
1441  GTATTCAGTTGTAATTGGAA CTTAGTCAACAGGCAGAATT TCTTAAGTAGTTCATGGGAT 1500
 108   V  F  S  C  N  W  N   L  V  N  R  Q  N  F   L  S  S  S  W  D  127
1501  GGATCTATAAAAATATGGTC CCCTCTAAGAAAGCAAAGTT TAATGACCCTTACTCCACGA 1560
 128   G  S  I  K  I  W  S   P  L  R  K  Q  S  L   M  T  L  T  P  R  147
1561  CCTTTAGAGATTACCAAAAT GGTGGATCCATTAAACGCCA TTATATTGAAAAAGAAAAGC 1620
 148   P  L  E  I  T  K  M   V  D  P  L  N  A  I   I  L  K  K  K  S  167
1621  TTTACAGGTATTTCAAAAAA CAGGAACTGTGTATACCAAG CACAGTTCTCGCCCCACGAC 1680
 168   F  T  G  I  S  K  N   R  N  C  V  Y  Q  A   Q  F  S  P  H  D  187
1681  CAAAATCTCGTATTATCCTG TTCAGGGAATTCTTATGCAA GCTTATTTGACATTAGACTA 1740
 188   Q  N  L  V  L  S  C   S  G  N  S  Y  A  S   L  F  D  I  R  L  207
1741  CCTTCCGGCAAAAATCAGAA TAATTTTTTAGTGCATTCAG GACTAGAAGCATTGACTTGC 1800
 208   P  S  G  K  N  Q  N   N  F  L  V  H  S  G   L  E  A  L  T  C  227
1801  GATTTCAACAAATACAGACC TTATGTAGTTGCCACAGGAG GTGTAGATAATGCCATTAGA 1860
 228   D  F  N  K  Y  R  P   Y  V  V  A  T  G  G   V  D  N  A  I  R  247
1861  ATCTGGGACATTAGGATGCT AAATAAAAATGAATCAGCGA CTATCAAGAGGACTGTGCCC 1920
 248   I  W  D  I  R  M  L   N  K  N  E  S  A  T   I  K  R  T  V  P  267
1921  GGCCAACTTCACAATTCATC ATGCATCAATGAAATCCCTA ATGCGCACGGGCTGGCAATC 1980
 268   G  Q  L  H  N  S  S   C  I  N  E  I  P  N   A  H  G  L  A  I  287
1981  AGAAAAGTTACCTGGTCCCC TCATCATTCCAATATTTTAA TGTCAGCTTCATATGATATG 2040
 288   R  K  V  T  W  S  P   H  H  S  N  I  L  M   S  A  S  Y  D  M  307
2041  ACCTGTCGAATATGGAGAGA TCTCAGCAACGATGGTGCAA AAGAAACATATAAAACTAAC 2100
 308   T  C  R  I  W  R  D   L  S  N  D  G  A  K   E  T  Y  K  T  N  327
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2101  TCTACGGATGCTACTAAAGG TTCCATTTTCAATTTCACAC AGCATTCAGAATTCGTATTT  2160
 328   S  T  D  A  T  K  G   S  I  F  N  F  T  Q   H  S  E  F  V  F   347
2161  GGAGCTGACTGGAGCTTGTG GGGAAAGCCAGGATATGTGG CTTCAACTGCATGGGATGGA  2220
 348   G  A  D  W  S  L  W   G  K  P  G  Y  V  A   S  T  A  W  D  G   367
2221  AATTTATTTGTATGGAACGG CTTAGGTTGACACATATTCT TTTTACACAAATTTTTTGCA  2280
 368   N  L  F  V  W  N  G   L  G  *                                  375
2281  TTATTTAAACTTTAAAATTA TTTTTCAATCATTGTCAGCA TTTGGCAGATCATTTACGTA  2340
2341  ATTATTTAATATTCTCAACA CGAAATTATGGGTAACAACG CTTCTGACTGTGCAAAAGAA  2400
2401  CATTCTAAAAACATACCTAC GGCAAAAACTGCTAATTACT CAAATTAATCAAGTTTTTAT  2460
2461  AACGACATAAGCGAGTGCTG AAAGAAGGAATATTTAGAGT CCGATTACCGTTTTATTAAT  2520
2521  AAACGACATCTTATATATTG TAAA  2544
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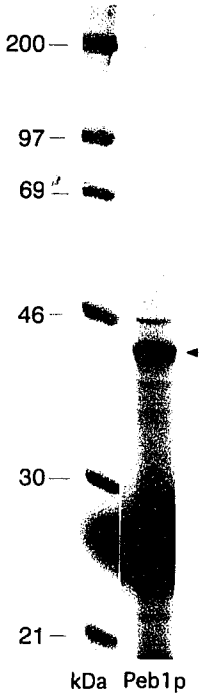


Figure 4-5 The in vitro expression of the PEB1 gene. pRSPEB1 was used for the in vitro transcription and translation. The Peb1p is indicated with an arrow. The big band at the 21 to 30 kDa range was due to the labeled Met-tRNA. ^{14}C-labeled molecular weight markers were used.

sequence, there is a sequence CGGTGGTTT (-222 to -214 upstream of ATG) (Fig. 4-4) which is consistent with the conserved sequence of the "peroxisome box" except one nucleotide (It is T at -222 position instead of A. Whether this not-fully conserved direct repeat on the minus strand of the PEB1 gene functions as a regulation element is unknown. Recently, Wang et al. (1992) have defined a negative regulating element in the 5′ UTR of the *S. cerevisiae* gene encoding AOx. The conserved sequence of this element is AGGGTAAT. No such sequence has been found in the 5′ UTR of this PEB1 ORF. a polyadenylation site (AATAAA, + 270 to 276 from the end of the ORF, see Fig. 4-4) was seen downstream of this ORF.

Genetic analysis of the cloned gene

Further experiments were carried out to test the authenticity of this ORF as the PEB1 gene, not a suppressor of the *peb1* mutant. The ORF was disrupted by one-step homologous recombination (Rothstein, 1991). As shown in Fig. 4-6, the majority of the ORF was replaced with the yeast TRP1 gene. The disruption of the genome was confirmed by PCR (Fig. 4-6*C*, see legend).

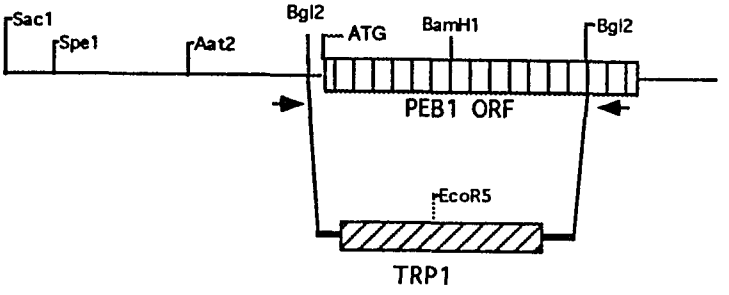
The phenotype of the knockout strain (JW75) was examined. It did not grow on a YNO plate (Fig. 4-9 *A*. sector 2). Immunofluorescence analysis with anti-thiolase showed cytosolic fluorescence (Fig. 4-9 *B* sector 2). When the knockout strain was subjected to digitonin permeabilization, it was found that the thiolase emerged with the cytosolic marker enzyme, phosphoglycerate kinase (PGK), whereas the catalase came out at higher digitonin concentrations (Fig. 4-10A). These results indicated that the knockout strain can package catalase, but not thiolase, into peroxisomes. This phenotype is identical with that of the peb1 mutant (see Chapters 2 and 3).

When the knockout strain was crossed with the *peb1* mutant, m6-D1, the formed diploid cells failed to package thiolase. The diploid was then sporulated and 4 tetrads were analyzed. All of the 4 tetrads showed a 0:4 pattern of thiolase packaging as tested by immunofluorescence with anti-thiolase. Based on this, I concluded

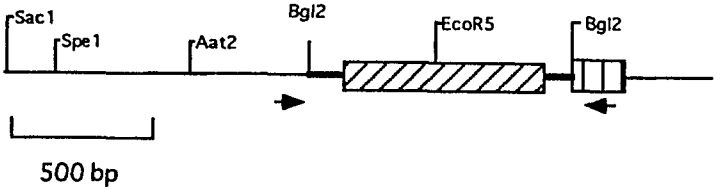
Figure 4-6 Disruption of the PEB1 gene. (A) The strategy to construct the disruption clone. The Bgl2 fragment of the PEB1 gene, which covers the majority of the PEB1 ORF (▭) was replaced by the TRP1 gene (▨). (B) The diagram of the expected disrupted genome in JW75. (C) Confirmation of the disruption. Two primers from the PEB1 gene, as indicated by arrows, were used for PCR amplification. The genomic DNAs prepared from wild type and JW75 were used as templates. The amplified fragment from JW75 is about 100 bp shorter than that from wild type cells and can not be digested by BamH1.

Disruption of PEB1 gene

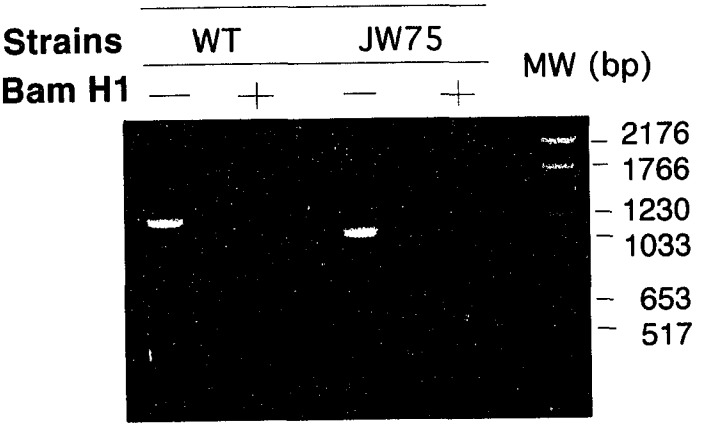
A.



B.



C.



that the cloned ORF is a bona fide PEB1 gene.

Structural features of the protein

The nucleotide sequence of the PEB1 gene was compared with the EMBL/GenBank nucleotide sequence data base (Dec 1993 release) with the FASTA search of the GCG sequence analysis program (version 7). No significant sequence similarity to any other known genes was found. When the deduced amino acid sequence was used to search the SwissPro protein data base (FASTA, GCG), the C-terminal two thirds of the protein shared some similarity with several yeast proteins, mainly STE4 (Whiteway et al., 1989), MSI1 (Roggieri et al., 1989) and PWP1 (Duronio et al., 1992). The regions of similarity with these genes were confined to the WD-40 motifs (also called β-transducin repeat) that all of these genes have (van der Voorn and Ploegh, 1992). The WD-40 repeat was first identified in a β-subunit of heterotrimeric G-protein which consisted of 7 such repeats in tandem (Fong et al., 1986). Proteins with more than two of these repeats have been classified into a family called the β-transducin

WD 40 repeat consensus	(n1)	LxGHxxxIxxΦxδ F L V	(n2)	ΦΦSGGxDxxΦxIWDδ TAA N C LFN S VY
(56- 90)	(56)	SfLTqdcLfdLaW	(neshenq)	VLVAQgDgtLrLFDT
(100-134)	(10)	FkEHereVisCnW	(nlvnrqn)	FLSSSwDgsIkIWSP
(190-204)	(66)			VLSCSgNsyAsLFDI
(215-250)	(11)	FlVHsglEalTcD	(fnkyrpyv)	VATGGvDnaIrIWDI
(279-313)	(29)	PnAHglaIrkVtW	(sphhsni)	LMSASyDmtCrIWRD
(338-373)	(15)	FtQHsefVfgAdW	(slwgkpgy)	VASTAwDgnLfVWNG

Φ. hydrophobic residues preferred. δ. non-charged residues.
x: any amino acid residues

Figure 4-7 Alignment of the six WD-40 repeats of Peb1p with the consensus WD-40 sequence. Numbers in parentheses on the left indicate the first and last amino acid residues of that repeat. The amino acid residues supposed to be conserved are capitalized. The actual conserved residues are capitalized and in bold type.

family (van der Voorn and Ploegh, 1992). The consensus sequence of this repeats is shown in Fig. 4-7. These repeat consists of 42-43 amino acids within which certain amino acid residues and their spatial relationship are highly conserved. The most conserved part is the second block. Using the MOTIF search of GCG sequence analysis program, two WD-40 repeats were found in Peb1p. Further comparison of the Peb1p sequence with the consensus sequence revealed four more repeats. This six repeats are located throughout the Peb1p except the first 56 residues. All the six repeats in Peb1p are highly conserved in the second block. They show considerable variability in the first block. Therefore, the Peb1p is a new member of the β-transducin family. Hydropathy analysis according to Kyte and Doolittle (Kyte and Doolittle, 1982) with a window size of 19 fails to identify any transmembrane domain.

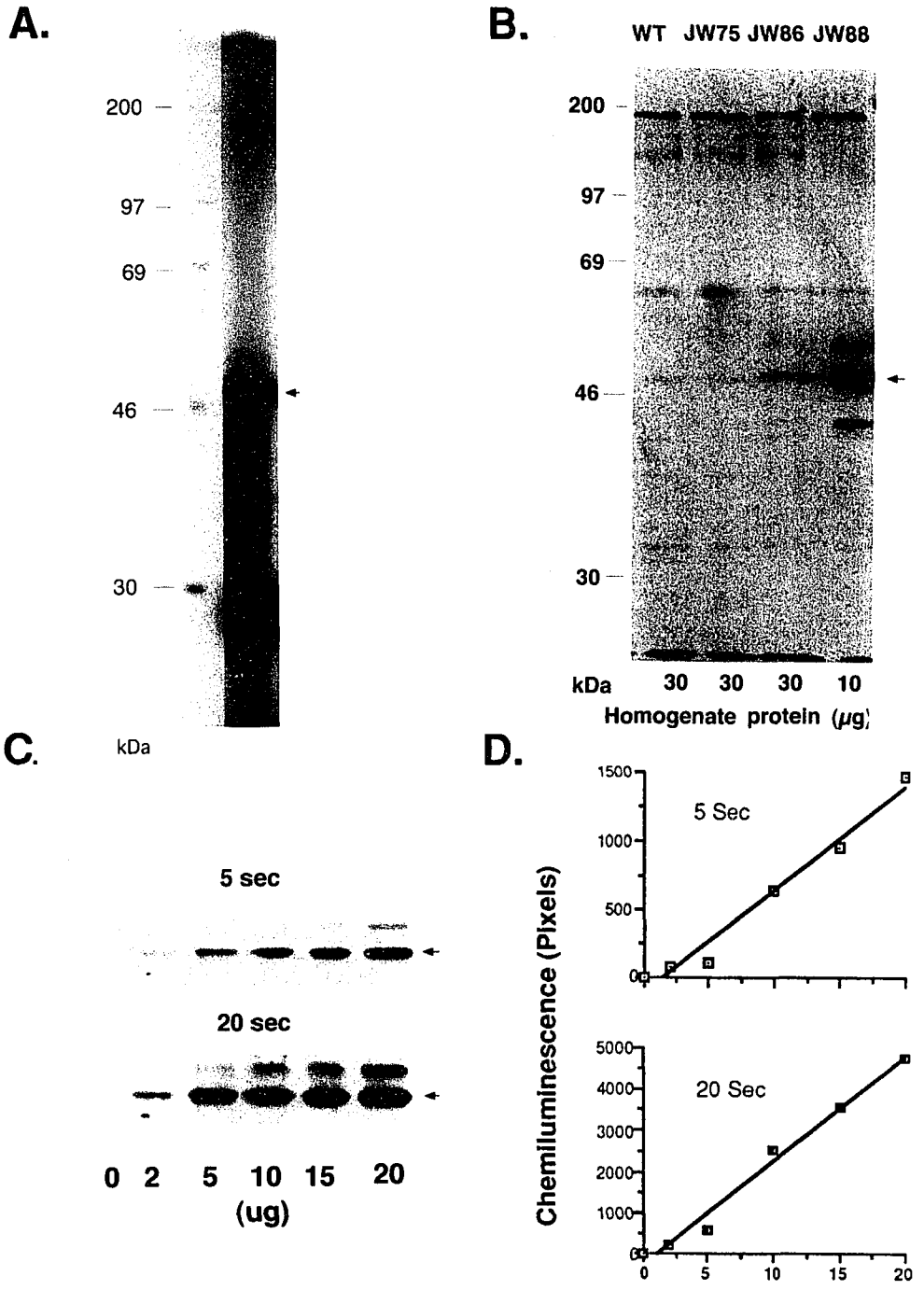
Identification of Peb1p

Epitope tagging was used for the surveillance of Peb1p. PCR amplification was used to add the 9 amino acid residue epitope (HA epitope, YPYDVPDYA) of hemagglutinin to the extreme C-terminal of

the PEB1 ORF (Wilson et al., 1984; Kolodziej and Young, 1991). This epitope can be recognized by a monoclonal antibody which bears the name 12CA5. When the PEB1 tagged with one copy of the epitope (PEB1HA) was expressed in yeast cells under its own promoter (1124 bp sequence of 5' upstream untranslated region), either in an integrating plasmid (Yip) or an episomal plasmid (Yep), no positive signal could be detected by western blot using antibody 12CA5. Both plasmids could restore the growth on YNO plates of the strain in which PEB1 was disrupted (JW75). It has been reported that sometimes one copy of the HA tag is not enough to be detected by monoclonal antibody 12CA5 but three copies are (Tyers et al., 1992). Therefore, a second PCR reaction was carried out to add two more copies of the HA epitope to the C-terminal of PEB1HA. When this triple-tagged PEB1 ORF (termed PEB1HA3, Fig. 4-1B) was expressed in an in vitro system, a band of about 48.5 kDa was detected (Fig. 4-8A which is 2.3 kDa bigger than the calculated molecular weight of the triple-tagged Peb1p (46.17 kDa). The cause for this difference

Figure 4-8 Expression and detection of the epitope-tagged Peb1p. *(A)* In vitro expression of the triple-tagged PEB1 as detected by fluorgraphy (arrow). The big band at the 21 to 30 kDa range was due to the labeled Met-tRNA. ^{14}C-labeled molecular weight markers were used. *(B)* In vivo expression of triple-tagged Peb1p. Glass bead homogenates from Wild type (WT), the PEB1 knockout (JW75), the knockout strain containing plasmid YipEB1HA3 (JW86) and the knockout containing plasmid pBXNPEB1HA3 (JW88) were separated by SDS-PAGE and immunoblotted with monoclonal antibody 12CA5 against the HA epitope and detected with chemiluminescence. *(C)* Effect of the amount of homogenate protein (2-20 μg) from the strain JW88 and the exposure time on the chemiluminescent signal from 12CA5. *(D)* Quantitation of the data of*(C)*.

Expression of the triple-tagged Peb1p



is not clear.

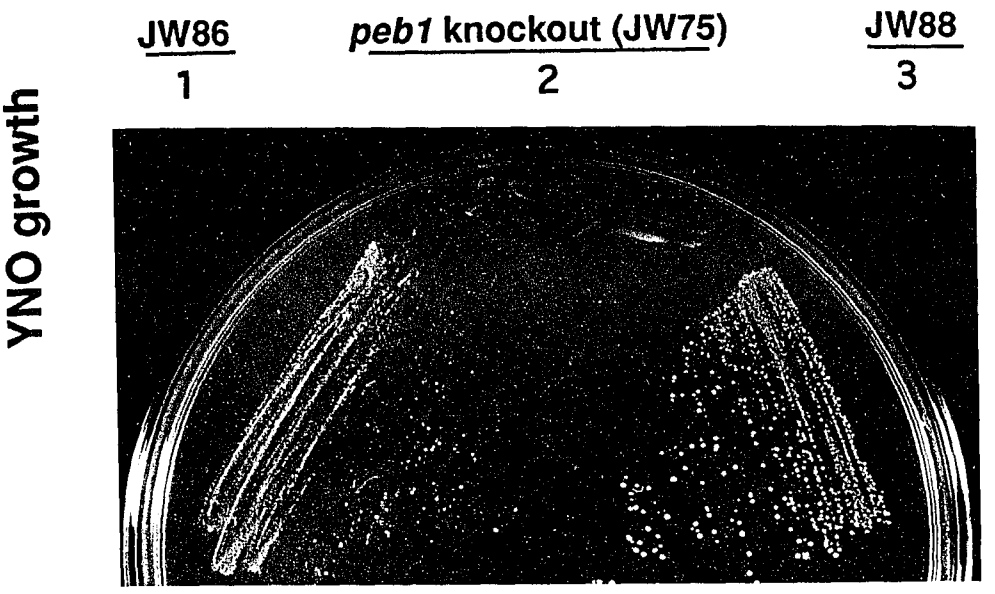
The triple-tagged PEB1 ORF with 1124 bp of 5'UTR was cloned in a Yip plasmid (YipPEB1HA3, Fig. 4-1C) which was transformed into the *peb1* null mutant after linearization in the TRP1 gene. The transformant was called JW86. The yeast transcription control elements are usually located within 500-600 bp of the 5' UTR (Guarente, 1984; Einerhand et al., 1991) . As JW86 contains only one copy of the triple-tagged PEB1 gene with 1124 bp of 5' UTR, its expression is assumed as wild type level. The triple-tagged PEB1 ORF was also cloned into an episomal plasmid under GAPDH promoter (pBXNPEB1HA3, Fig. 4-1D) which was transformed into the *peb1* null mutant, generating a new strain JW88. The GAPDH promoter is a strong constitutively expressed promoter (Bitter and Egan, 1984; Schild et al., 1990). As the triple-tagged PEB1 gene is under the control of such a strong promoter and has multiple copies in JW88, its expression level in this strain will be very high. The yeast glass bead lysates of wild type, JW75, JW86 and JW88 after growth in glycerol plus oleate medium were blotted with antibody 12CA5. As

shown in Fig. 4-8 *B* , a faint band at molecular weight of about 48.5 kD, same as the in vitro translation product, was detected in JW86, but not in wild type or the knockout (JW75). In JW88, a major band at 48.5 KDa position with much higher intensity was detected. Based on this, I conclude that the 48.5 KDa protein is the triple-tagged Peb1p. Two less intense bands, one above the major band and one below it, also can be seen in JW88. The lower band probably is the degradation product. The nature of the upper band remains to be tested.

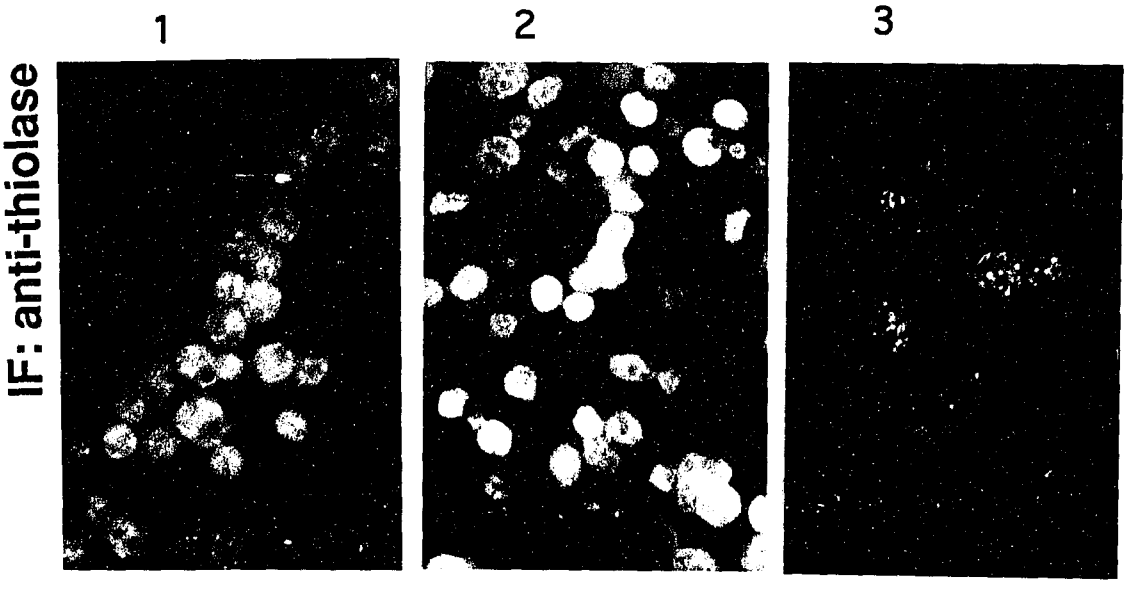
It was found that the presence of the tagged protein in the *peb1* knockout strain did not affect its growth either in YPD or in YPGO medium (data not illustrated). Further experiments were carried out to test if the triple-tagged PEB1 can complement the *peb1* null allele. As shown in Fig. 4-9A, both JW86 and JW88 can grow on a YNO plate. However, the colony size of JW86 was smaller than that of JW88. With immunofluorescence using antiserum

Figure 4-9 Complementation of the *peb1* null mutant with the triple- tagged PEB1 gene. *(A)* Growth on an oleate medium of JW86 (1), JW75 (2) and JW88 (3). *(B)*. Immunofluorescence analysis of (1), (2), (3) with anti-thiolase.

A.


JW86
1
peb1 knockout (JW75)
2
JW88
3
YNO growth

B.


1
2
3
IF: anti-thiolase

against thiolase, JW86 showed punctate staining and pretty strong cytosolic fluorescence; while JW88 had punctate labelling with little cytosolic fluorescence (Fig. 4-9B). When the wild type copy of the PEB1 gene with 1124 bp sequence of 5' upstream untranslated region was cloned in an integrating plasmid, the transformant showed wild type fluorescence pattern with anti-thiolase (data not illustrated). These results indicated the triple-tagged PEB1 gene can only partially complement the *peb1* null mutant when it is expressed under its own promoter; Overexpression of this gene under the strong constitutive GAPDH promoter can restore the punctate fluorescence with anti-thiolase and the growth on YNO medium to wild type.

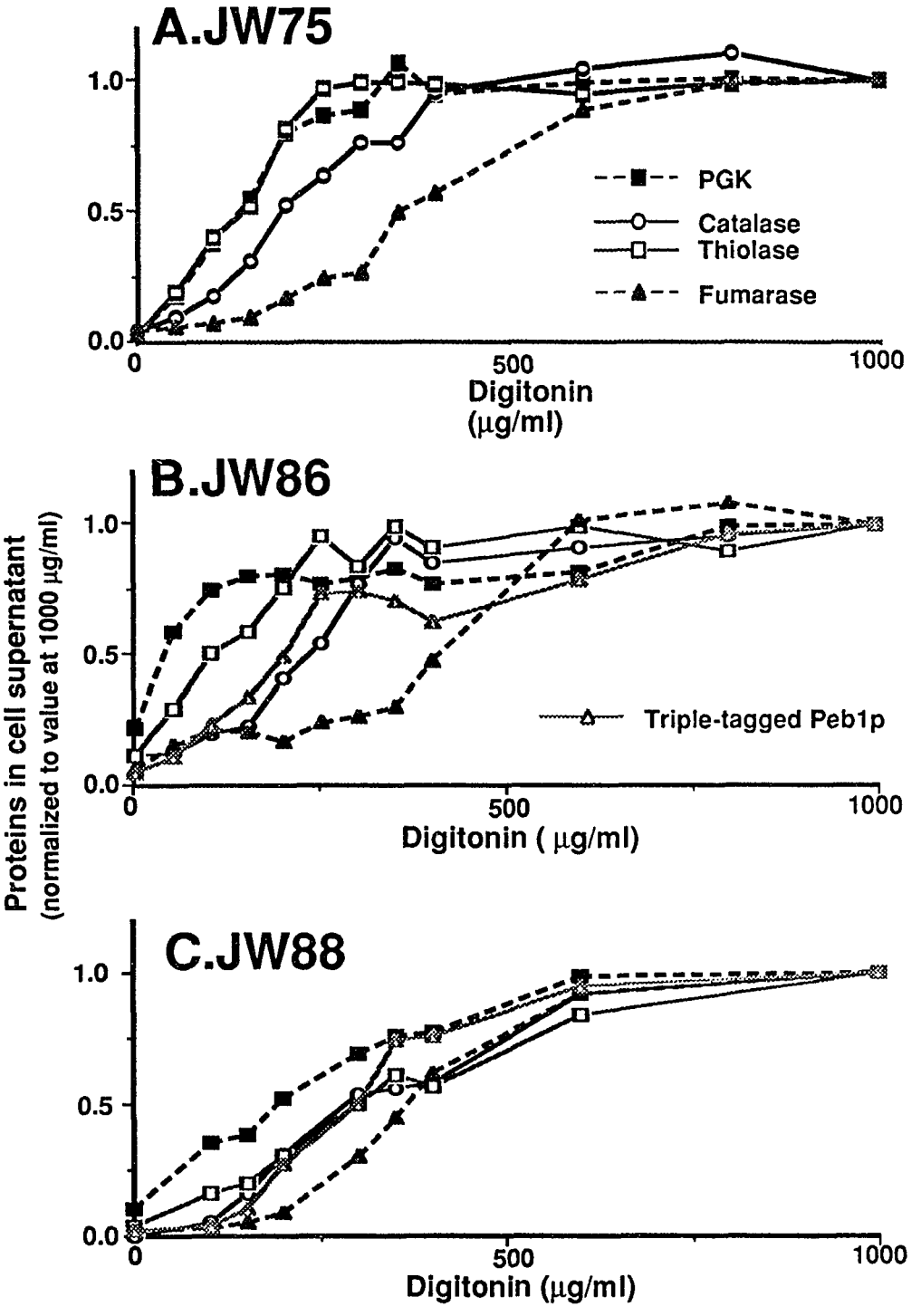
Subcellular location of Peb1p

Three different techniques were employed to determine the subcellular location of Peb1p, both at its normal expression level and when overexpressed.

Digitonin permeabilization: Strain JW86 and JW88 were grown

in glycerol and oleate medium, converted to spheroplasts and permeabilized with different concentrations of digitonin. The release of proteins upon increasing digitonin concentration was plotted in Fig. 4-10*B and* C). In both strains, the digitonin concentration needed to release the cytosolic protein (PGK), to release the peroxisomal catalase A, and to release the mitochondrial protein (fumarase) were generally similar to those required in the *peb1* knockout strain. In both strains, the release pattern of triple-tagged Peb1p upon different concentrations of digitonin was the same as that of catalase, suggesting that this protein is located in the peroxisomes in both strains. The digitonin required to release thiolase was different in these two strains. In JW86, the digitonin concentration required for the release of thiolase was between that of PGK and catalase. In light of the strong cytosolic fluorescence and punctate staining seen in these cells by immunofluorescence with anti-thiolase (Fig. 8B), this pattern can be explained as the dual localization of thiolase in both cytosol and peroxisomes

Figure 4-10 Digitonin cell fractionation. Aliquots of spheroplasts were incubated with digitonin at different concentrations for 20 min at 4° C, and centrifuged to pellet the cells. The supernatants were analyzed for the release of proteins. The activity of catalase (circle) and fumarase (filled triangle) were assayed enzymatically. The PGK (filled square), thiolase (square) and triple-tagged Peb1p (triangle) were analyzed by immunoblotting. Cells are from *(A)* JW75, *(B)* JW86 and *(C)* JW88.

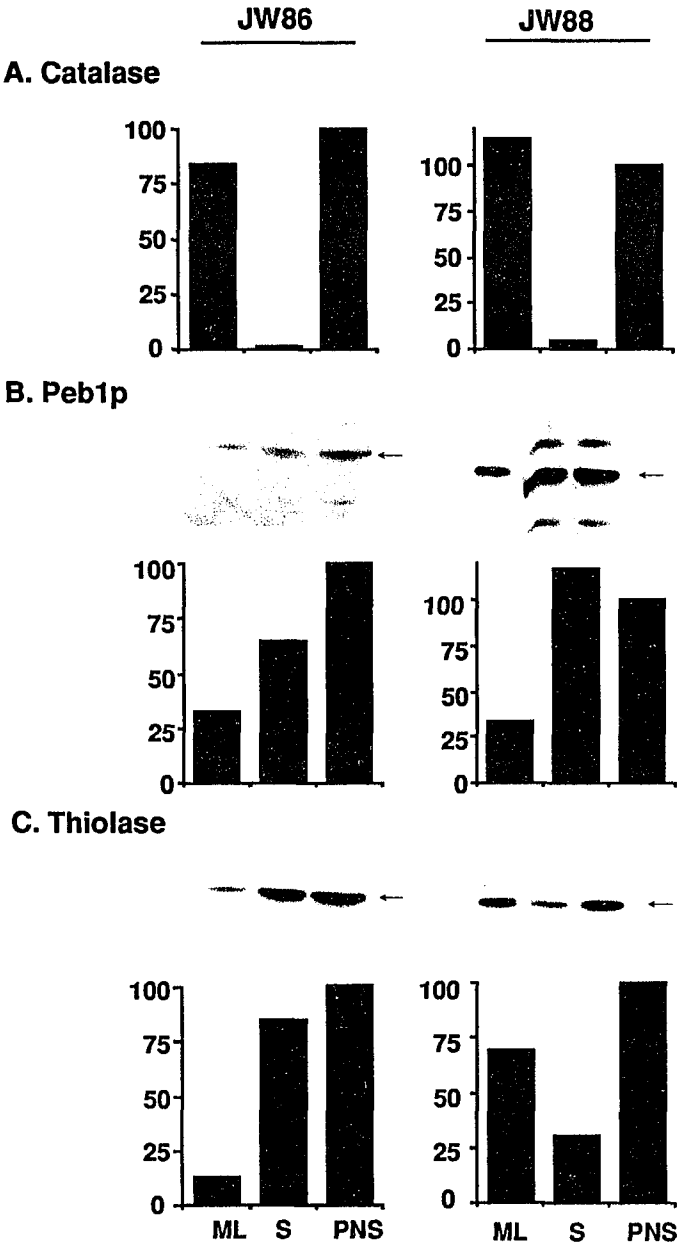

A.JW75
1.0
0.5
0.0
0
500
1000
Digitonin
(μg/ml)
PGK
Catalase
Thiolase
Fumarase
B.JW86
Proteins in cell supernatant
(normalized to value at 1000 μg/ml)
1.0
0.5
0.0
0
500
1000
Digitonin (μg/ml)
Triple-tagged Peb1p
C.JW88
1.0
0.5
0.0
0
500
1000
Digitonin (μg/ml)

in this strain. In JW88, the release pattern of thiolase is similar to that of catalase except at 100 μg of digitonin point. At that point about 15% of thiolase was already in the supernatant, while little catalase was out. This result indicates that the majority, if not all, of the thiolase in JW88 is located inside the peroxisome.

Cell fractionation: The yeast cells of strain JW86 and JW88 grown in glycerol plus oleate medium were converted to spheroplasts. The spheroplasts were homogenized to break the plasma membrane. The homogenates were centrifuged at 1,500 g for 5 min to remove the unbroken cells and nuclei. The supernatant, which is termed the post-nuclear supernatant, was fractionated by differential centrifugation at 25,000 g for 15 min to spin down the mitochondria and peroxisomes. The distribution of catalase, thiolase and tagged Peb1p in the supernatant and pellet in these two strains was analyzed (Fig. 4-11*A, B, C*). The pellet fraction (also called ML fraction) consisted mainly of mitochondria and peroxisomes.

Figure 4-11 Differential centrifugation of yeast lysates. The post-nuclear supernatants (PNS) of JW86 and JW88 were fractionated into a 25,000 g pellet (ML) and supernatant (S). The distribution of catalase, Peb1p and thiolase in these three fractions was analyzed. For Western blot detection, equal percentage of PNS, ML and S was loaded on each lane. In the plot, the amount in the PNS was normalized to 100% (Y-axis). *(A)* Distribution of catalase. *(B)* Distribution of triple-tagged Peb1p. Top: immunoblot analysis, raw data. Bottom: quantitation by densitometry. *(C)* Distribution of thiolase. Top: Immunoblot, raw data. Bottom: quantitation by densitometry.

Differential centrifugation



In both strains, catalase was mainly found in the pellet fraction, consistent with its peroxisomal location. The majority of thiolase was found in the pellet in JW88. About 30% of thiolase was in the supernatant. In JW86 the majority was seen in the supernatant. In both strains, about 30% of Peb1HA3 was recovered in the pellet.

The pellet fraction was loaded on the linear Nycodenz gradient for the separation of peroxisomes and mitochondria. After the centrifugation, the fractions were assayed for catalase activity as peroxisome marker enzyme and cytochrome C oxidase as the mitochondrial marker enzyme. The fractions were further analyzed by Western blotting with antiserum against thiolase and monoclonal antibody 12CA5 which recognizes the HA epitope tag on Peb1p. As shown in Fig. 4-12 and 4-13, the peroxisomes, as marked by catalase activity, and mitochondria are well separated in these two preparations. In both cases, the most intense bands of tagged Peb1p, and the thiolase, appeared in fractions where the catalase peak activity was present. In the gradient prepared from JW88, the

Figure 4-12 Nycodenz gradient fractionation of JW86. *(A)* Distribution of catalase, cytochrome C oxidase and proteins in the gradient. The ordinate represents relative concentrations as calculated according to de Duve (1967). The relative concentration equals the absolute concentration in the fractions divided by the concentration that would have been found if the enzyme was distributed uniformly through the gradient. The X-axis indicates the fraction number. *(B)* Distribution of triple-tagged Peb1p and thiolase in fractions 3-14. 10 µl of each fraction was separated by SDS-PAGE. One membrane (the top, which was run on a smaller gel) was blotted with antibody 12CA5. Another membrane (the bottom) was blotted with anti-thiolase. Chemiluminescence was used for the detection.

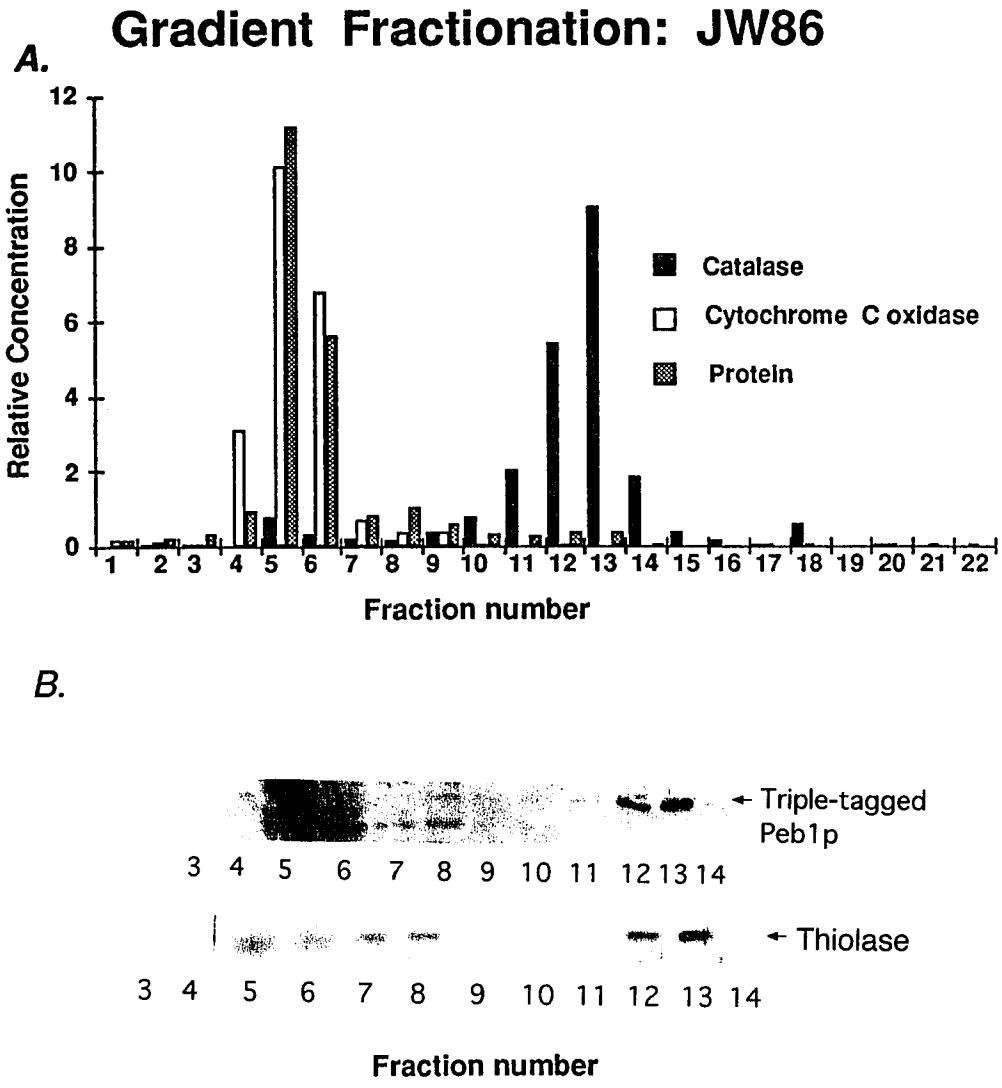
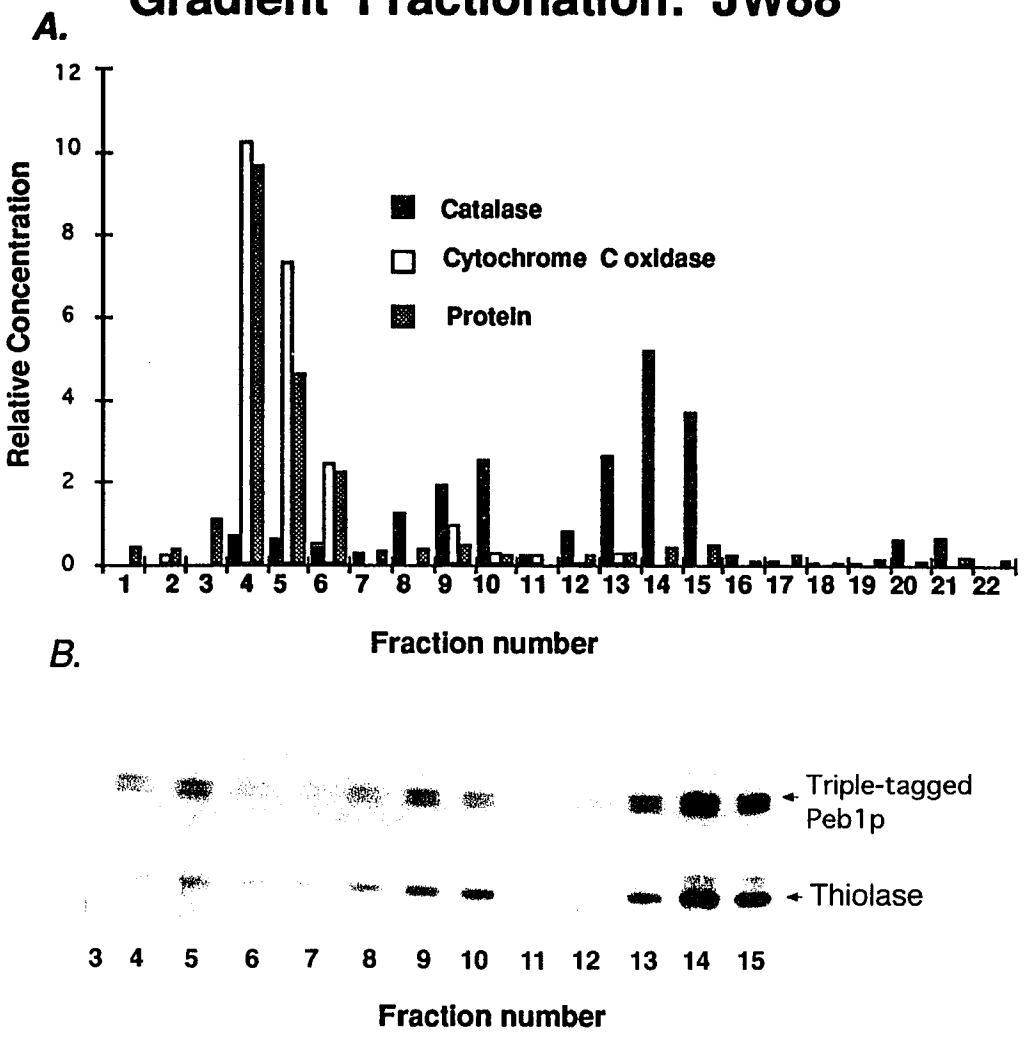

Gradient Fractionation: JW86
A.
Relative Concentration
0
2
4
6
8
10
12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Fraction number
Catalase
Cytochrome C oxidase
Protein
B.
Triple-tagged Peb1p
3 4 5 6 7 8 9 10 11 12 13 14
Thiolase
3 4 5 6 7 8 9 10 11 12 13 14
Fraction number

Figure 4-13 Nycodenz gradient fractionation of JW88. *(A)* Distribution of catalase, cytochrome C oxidase and proteins in the gradient. The ordinate represents relative concentrations (see Fig. 4-12 legend for details) and the X-axis indicates the fraction number. *(B)* Distribution of triple-tagged Peb1p and thiolase in fractions 3-15. 10 µl of each fraction was separated by SDS-PAGE, blotted sequentially with antibody 12CA5 and then anti-thiolase and detected with chemiluminescence.


Gradient Fractionation: JW88
A.
Relative Concentration
12
10
8
6
4
2
0
Catalase
Cytochrome C oxidase
Protein
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Fraction number
B.
Triple-tagged Peb1p
Thiolase
3 4 5 6 7 8 9 10 11 12 13 14 15
Fraction number

peroxisomes had two peaks, with a smaller peak in fractions 8 to 9 and a larger peak in fractions 13 to 15 (Fig. 4-13). The triple-tagged peb1p and thiolase also showed two peaks in the same positons. This result indicated that the triple-tagged Peb1p and thiolase in the ML fraction was mainly located in peroxisomes in both strains. A small amount of this triple-tagged Peb1p and thiolase was also seen in the mitochondria peak fractions, which might be due to the contamination of peroxisomes. The fact that the majority of the triple-tagged peb1p was present in the 25,000 g supernatant in both expression levels will be discussed later. It was noticed that in fractions from JW88 a lower molecular weight band had been detected by monoclonal antibody 12CA5, in addition to the major band of 48.5 kDa. The reason for the appearance of this band is not clear. One possible explanation would be due to the degradation of triple-tagged Peb1p during the storage in the freezer. This extra band was not detected from fractions from JW86.

The gradient results also indicate that the thiolase in the ML fraction of both expression levels is located in the peroxisomes.

However, the majority of thiolase in JW86 is found in the 25,000 g supernatant. It also seems unlikely that this protein is preferentially leaked out of the organelle in JW86 as the thiolase was mainly found in the ML pellet fraction in JW88, the same strain but with overexpression of the triple-tagged Peb1p. In light of the immunofluorescence results with anti-thiolase and digitonin permeabilization of this strain, this result suggests that the thiolase is partially packaged in JW86. For JW88, the majority of thiolase was found in the ML fraction and peroxisomes. about 30% of thiolase remained in the supernatant.

Immunofluorescence: The subcellular location of Peb1p was further studied by immunofluorescence analysis using antibody 12CA5. As shown in Fig. 4-14, both JW86 and JW88 had punctate staining. The number of stained particles was more in JW88 than that in JW86. The cytosolic fluorescence in JW88 was somewhat stronger than that in JW86. As a control, the *peb1* knockout strain JW75 had very weak, if any, staining. In light of the results obtained by digitonin titration and cell fractionation, I conclude that

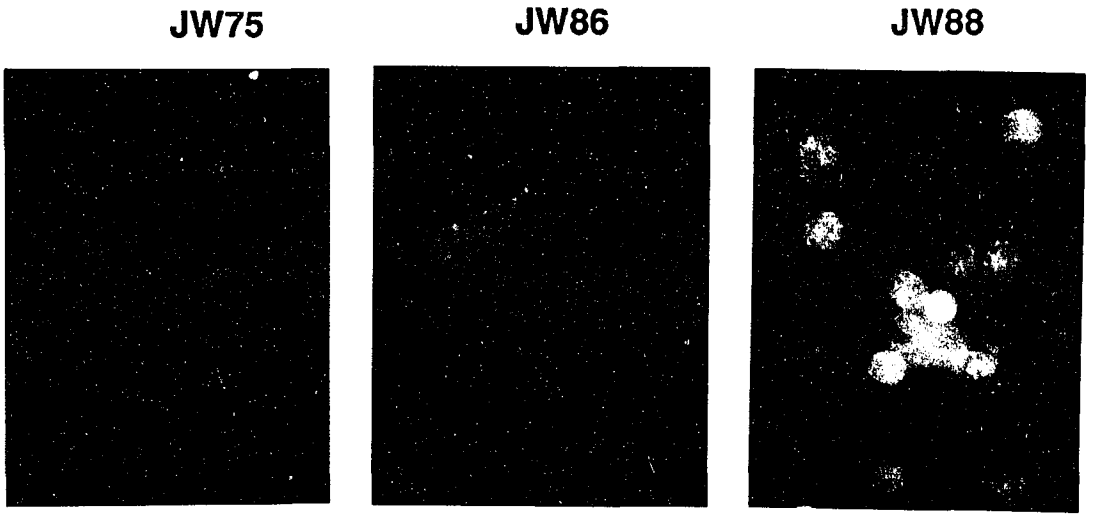


Figure 4-14 Immunofluorescent detection of triple-tagged Peb1p. Immunofluorescence analysis of JW75, JW86 and JW88 with antibody 12CA5.

these particles are stained peroxisomes.

Intraperoxisomal location of Peb1p: Protease K treatment

The above studies demonstrated that the Peb1p is peroxisome associated. To further test the location of this protein in relation to the peroxisomal membrane, a protease K digestion experiment was carried out. The 25,000 g pellet fraction of JW88 was digested with agarose bead-coupled protease K for different lengths of time, in the absence and presence of 0.1% Triton X-100. The digestion of thiolase, which is a peroxisomal matrix protein and was used as a control in this experiment, and tagged Peb1p was analyzed by Western blot. As seen from Fig. 4-15, little digestion occurred in the absence of detergent for both proteins. These two proteins were digested only when the organelles were lysed with Triton X-100. The rate of digestion for thiolase was much faster than that for tagged peb1p. This result demonstrated that the tagged Peb1p, like the thiolase, was protected by the peroxisomal membrane, suggesting that the Peb1p is likely located inside the peroxisomal membrane.

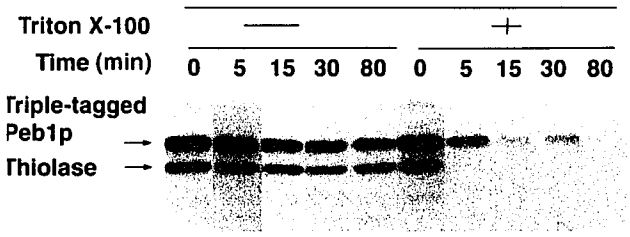


Figure 4-15 Protease K treatment of organelles. The 25,000 g pellet fraction from JW88 was resuspended in 1.2 M sorbitol, 5 mM MES, pH 6.0. Aliquots of the fraction were treated with protease K coupled with agarose beads (25 mg/ml) for different amounts of time, in the absence (-) and presence (+) of 0.1% Triton X-100 at room temperature. The digestion products were separated by SDS-PAGE and blotted with 12CA5 and anti-thiolase consecutively.

Regulation of PEB1 expression by carbon source

The expression of the PEB1 gene in cells grown in different carbon sources was studied in the following experiments. The upstream 750 bp sequence of the PEB1 gene was fused in frame with the bacterial LacZ gene on an episomal plasmid (Fig. 4-1E). This construct was transformed into wild type yeast. The transformant (JW72) was grown in different growth conditions. Glass bead homogenates were prepared for the analysis of the expression of β-galactosidase, which is under the control of the PEB1 promoter, catalase A, thiolase and PGK. As shown in Fig. 4-16A, the β-galactosidase, which was detected by Western blotting, was present in very low level when cells were grown in glucose. Its expression was increased when cells were grown in glycerol. There was no further increase when oleate was included in the glycerol medium. The same membrane was reblotted with antiserum against PGK, the expression of which is independent of carbon sources. The PGK was

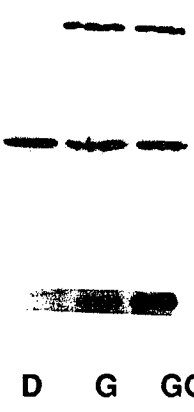
Figure 4-16. PEB1 promoter-dependent expression of β-galactosidase. Glass bead homogenates from JW72 grown in glucose (D), glycerol (G) and glycerol plus oleate (GO) were assayed for the expression of β-galactosidase, PGK and catalase. (*A*) Western blot detection of β-galactosidase. (*B*) Western blot detection of PGK. (*C*) Western blot detection of thiolase. (D) Catalase activity. Catalase A is the only active isoenzyme in this strain. The level of the catalase of cells grown in glycerol plus oleate (GO) was normalized to 100%. Catalase activity was undetectable when cells were grown in glucose.

PEB1 promoter dependent expression of the β-galactosidase

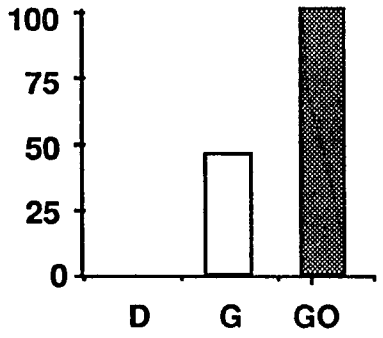
A. β-galactosidase

B. PGK

C. Thiolase



D. Catalase



almost equally present under these three growth conditions (Fig. 4-16*B*). The thiolase, which was detected also by Western blotting, was barely detectable in glucose, present in moderate amount in glycerol and induced when oleate was present in the glycerol medium (Fig. 4-16*C*). The expression of catalase A, which is the only active catalase in this strain, behaved like thiolase. (Fig. 4-16*D*).

Further evidence concerning the regulated expression of PEB1 comes from the Northern blot analysis (Fig. 4-17). Using a PEB1 fragment as probe, a very faint band was detected from cells grown in glucose. The intensity of this band was much higher when cells were grown in glycerol. There appeared to be a small further increase in band intensity when cells were grown in glycerol plus oleate. As a control, the same membrane was probed with a fragment of the TCM1 gene which encodes the ribosomal protein L3 (Schutz and Fressen, 1983). This band showed a similar relative intensity in glycerol compared to glycerol plus oleate as did PEB1. The TCM1 band is much more intense from cells grown in glucose

than those from cells grown in glycerol and glycerol plus oleate. The intensity difference of TCM1 messenger in these three conditions probably reflects the small difference of growth state of cells. It have been reported very recently (Ju and Warner, 1994) that the expression of ribosomal protein messengers are very sensitive to the growth states of cells. The transcription of ribosomal protein genes decreases and disappears at an unexpectedly early stage in the growth cycle when the cells are still in the log phase. In this experiment, even though the growth of cells in these three growth conditions was in log phase, the stage of log phase could be different. Nevertheless, this control indicated that the messenger RNA in the sample from glucose culture was present and intact. The transcriptional expression of the PEB1 gene is consistent with that of β-galactosidase under the control of PEB1 promoter as detected by Western blot. Based on these data, I conclude that the expression of PEB1 is repressed in glucose and can be derepressed by glycerol. Oleate may not further induce its expression, based on the β-galactosidase data (Fig. 4-16). These data appear to be more reliable because they have a good PGK control on the same blot.

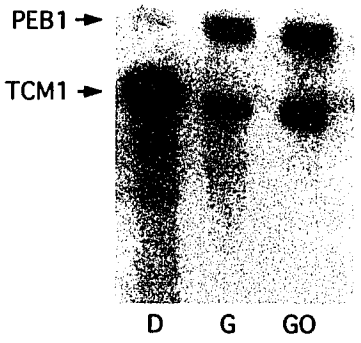


Figure 4-17. Northern blot of PEB1 gene expression. Total RNAs were prepared from wild type cells grown in glucose (D), glycerol (G) and glycerol plus oleate (GO) medium and probed with the Aat2 fragment of the PEB1 gene (PEB1) and the Hpa1-Sal1 fragment of the TCM1 gene (which encodes the ribosomal protein L3) (TCM1) consecutively.

Discussion

I have cloned the PEB1 gene by complementing the *peb1* mutant with a yeast genomic library. The authenticity of the PEB1 gene was confirmed by gene disruption and genetic comparison of the knockout with the original mutant. This gene is not essential for growth on glucose, but is required for growth on oleate and import of thiolase.

Peb1p is a member of the β-transducin family

The sequence analysis of Peb1p reveals that it contains six WD-40 repeats (or β-transducin repeats), putting this protein into the family of β-transducin (van der Voorn and Ploegh, 1992). Proteins from this family are involved in diverse cellular pathways, including signal transduction (Gβ subunit) (Fong et al., 1986), (STE4) (Whiteway et al., 1989), cell division (CDC4) (Peterson et al., 1984; Fong et al., 1986) , gene regulation (TUP1) (Williams and Trumbly, 1990) and RNA processing (PRP4) (Banroques and Abelson, 1989). The WD-40 repeats in these proteins are proposed to be involved in protein-protein interaction (van der Voorn and Ploegh, 1992). Many

of the genes encoding proteins in this family have been found to be functionally related to members of another family of proteins called TPR family (Goebl and Yanagida, 1991). Proteins in this family contains multiple repeats which consist of highly degenerate 34 amino acid residues. Examples of these functionally related pairs from these two families are SSN6 (CYC8)/TUP1(Williams et al., 1991) which are involved in gene regulation, PRP4/PRP6 which are involved in RNA processing, and CDC 20/CDC16, CDC23 which are involved in cell division (see review, (Goebl and Yanagida, 1991)). One pair of these proteins, SSN6 (CYC8)/TUP1, has been shown to be physically associated with each other (Williams et al., 1991). It is thought by some that the proteins from these two family might interact with each other (Goebl and Yanagida, 1991). The recently cloned PAS8 gene in *Pichia pastoris* (McCollum et al., 1993) and its homologue in *S. cerevisiae* (PAS10 (Van Der Leij et al., 1993)) contain seven and eight TPR motifs respectively. Pas8p/Pas10p is required for the import of peroxisomal proteins with the SKL signal but not thiolase. This function is complementary with that of Peb1p. Pas8p in *P. pastoris* has been shown to be associated with the

peroxisome membrane, but the topology of this protein in relation to the membrane is not clear. It will be interesting to test the speculation that the Pas10p and Peb1p might physically interact with each other.

Peb1p is a peroxisomal protein

In this study, epitope tagging was used to study the subcellular location of Peb1p. With the digitonin permeabilization fractionation method, the triple-tagged PEB1 gene product was found to be mainly located in peroxisomes, under either normal or overexpression conditions. When analyzed with gradient fractionation, the Peb1p in the ML fraction colocalized with other peroxisomal proteins at both expression levels. However, when analyzed by differential centrifugation, the majority of this protein was present in the 25, 000 g supernatant where the cytosolic proteins are supposed to be located. One explanation of these results is that this protein has a dual location, both in the cytosol and peroxisomes. Digitonin permeabilization results do not support this explanation. The alternative explanation, which I think more likely, is that Peb1p is

located in peroxisomes. The presence of this protein in the cytosol fraction in differential centrifugation might be an artefact, due to the release of Peb1p from the damaged organelle. Previous studies have shown that the peroxisome is very fragile and tends to be damaged during homogenization, centrifugation and resuspension of the fractions, causing the release of peroxisomal proteins (Thieringer et al., 1991; McCammon et al., 1990b) . Different peroxisomal proteins have different sensibility to the damage in terms of leakage. In McCammon et al. (1990b)'s study, about 67% of the thiolase was found in the 25,000 g pellet. However, only 13% of malate synthetase, which ends with SKL and has been shown in peroxisomes by immuno-gold labelling, was present in the pellet. Alexson et al. (1985) have also observed differential release of rat liver peroxisomal proteins when the organelle was damaged by various methods. The Peb1p probably behaves like malate synthetase, being very sensitive to the damage of peroxisomes occurring during the homogenization, centrifugation and resuspension. As a contrast, the digitonin cell fractionation is based on the molecular interaction between the digitonin and sterols

in the membrane. No physical damage to the organelle will happen during this process. Digitonin fractionation should reflect more truthfully the physiological location of the protein. However, from the data I can not exclude the possibility that some of the Peb1p is located in the cytosol.

The triple-tagged Peb1p is only accessible to protease K when the organelle is broken with Triton X-100 (Fig. 4-15). Sequence analysis fails to detect any trnasmembrane domain in this protein. Based on these results, It seems likely that this protein is located inside the peroxisome membrane. Whether it is a matrix protein or peripheral membrane protein remains to be tested.

When the triple-tagged Peb1p at the extreme C-terminal was expressed under its native promoter, it only could partially complement the *peb1* null mutant (Fig. 4-9). The wild type copy of the gene constructed in the same vector (pJW42, Table 4-2) could fully restore the function of the *peb1* null mutant. These results indicate that the addition of the HA epitope on the C-terminus of the Peb1p decreased its function. This phenomenon has been seen in

other proteins with epitope tagging (Elion et al., 1993). As the triple-tagging of Peb1p did not result in an SKL-type sequence at the C-terminus of the protein, it is unlikely that the peroxisomal location of the tagged Peb1p is due to the tagging. Therefore, the targeting information in the tagged protein must residue in the Peb1p sequence. Sequence analysis of the Peb1p demonstrates that almost the whole Peb1p but the N-terminal 56 residues consists of six WD-40 repeats. Then it is possible that the targeting information is located in this N-terminal region. Comparison of the N-terminal sequence with that of mammalian peroxisomal thiolase reveals no obvious homology. Further experiments are required to test the location of the targeting signal in Peb1p.

The function of Peb1p in peroxisome biogenesis

In our collection of *peb* mutants, two groups are selectively defective in the import of one subset of peroxisomal enzymes. The *peb1* mutant can not package thiolase, but the import of catalase and

acyl-CoA oxidase is normal. The *peb5* mutants fail to package catalase, but have normal thiolase and AOx packaging. These three proteins are thought to have different targeting information. The catalase has internal targeting sequence in addition to the C-terminal SKL-like signal (Kragler et al., 1993). The fact that the *pas8* mutant in *P. pastoris* (McCollum et al., 1993) (which is likely to be the equivalent of *pas10* in *S. cerevisiae* (Subramani, 1993)) fails to import catalase and proteins with the SKL signal suggests that catalase might use the C-terminal SKL-like signal for its import in vivo. The *S. cerevisiae* thiolase probably uses N-terminal sequence as the targeting signal like its mammalian counterpart (Osumi et al., 1991; Swinkels et al., 1991). The topogenic sequence in AOx has not been defined yet. As it lacks the two signal sequences described above, it probably uses an the internal targeting sequence as its counterpart does in *C. tropicalis* (Small et al., 1988). Based on these facts, I have proposed in Chapter 3 that three pathways or three branches of a pathway, each represented by one of these three peroxisomal proteins, are likely involved in peroxisomal protein import. As the *peb1* mutant affects only the

packaging of thiolase, the Peb1p is likely to be a component of the import machinery specific for thiolase and other peroxisomal proteins using the same targeting signal. One obvious role of Peb1p in thiolase import is as a receptor, decoding the thiolase-type topogenic information. This function would require that the Peb1p be exposed to the cytosol side of the peroxisome, or be located in the cytosol. As the data suggests the Peb1p is located inside the peroxisomal membrane, this role seems unlikely. Other role(s) for the Peb1p have to be sought.

Based on the location of Peb1p and the phenotype of the *peb1* mutant, I propose that Peb1p could work as an intra-organelle receptor for thiolase import. Conceivably, thiolase could be recognized by a receptor in the cytosol or the peroxisome membrane. This recognition could result in the insertion of the polypeptide through the peroxisome membrane. The Peb1p in the peroxisomes could recognize the thiolase peptide as it is inserted. The correct interaction of Peb1p with peptide could initiate a series of protein-protein interactions involving Peb1p which result in the "pulling" of

the thiolase into the peroxisomes. The proposed role of Peb1p in thiolase import is somehow similar to that of the mitochondria Hsp70p (SSC1 gene product) in mitochondrial import (Craig et al., 1989; Stuart et al., 1994). Two predictions can be made according to the proposed import model of thiolase. First, there would be a receptor(s) outside the peroxisome membrane which recognizes thiolase. Second, the Peb1p would specifically interact with thiolase, presumably through thiolasae's topogenic sequence. These speculations about the role of Peb1p in thiolase import can be tested in future studies.

The regulated expression of Peb1p

By the gene fusion study and Northern blots, I have shown that the expression of the PEB1 gene was repressed in glucose and derepressed in glycerol (Fig. 4-16, 4-17). Based on the better-controlled gene fusion study (Fig. 4-16), the presence of oleate in glycerol medium appears not to further induce its expression. This pattern is different from the expression of thiolase and catalase. For these two peroxisomal proteins, their expression was repressed

in glucose, derepressed in glycerol and further induced when oleate is present. This not-fully-coordinate expression of Peb1p and thiolase might be due to the nature of the role of Peb1p. Peb1p, as a component in thiolase import, would only transiently interact with thiolase and other peroxisomal proteins with similar targeting signal. This interaction would be similar to the enzyme-substrate interaction in that small amount of Peb1p can facilitate the import of a lot of thiolase. When cells are grown in glucose, the expression of thiolase and other peroxisomal proteins is repressed, and so is the Peb1p's as it is not needed. As cells are shifted to glycerol, the expression of thiolase and other peroxisomal proteins is increased, so is the expression of Peb1p as the need for this protein is increased. When the oleate is present in the glycerol medium, the peroxisomal proteins are further induced. As the level of Peb1p due to the derepression is enough to accommodate the increased thiolase in this condition, there is no need for its further induction.

Chapter 5

Summary and Future Studies

In eukaryotic cells, proteins are sorted and segregated into subcellular compartments. The peroxisome is one such compartment. Proteins destined to peroxisomes have topogenic information which resides on the polypeptide. A complex machinery is responsible for the assembly of the peroxisome and/or the import of proteins. In this thesis, I reported the study of this process at the molecular level in *S. cerevisiae*.

In Chapter 2, I described the development of a positive selection procedure, which exploited the toxicity of H_2O_2, to isolate peroxisome biogenesis (*peb*) mutants. This selection procedure led to the identification of 5 *peb* complementation groups. Four of these groups are distinct from the 6 groups of peroxisome assembly (*pas*) mutants isolated by Erdmann et al. (1989, Kunau and Hartig, 1992). One mutant from each group was analyzed by immunofluorescence, electron microscopy, immunoelctron microscopy and electron

microscopic cytochemistry-DAB staining. One group appears to have fewer peroxisomes and express reduced amounts of peroxisome proteins. Two groups do not have recognizable peroxisomes under EM, resembling human Zellweger syndrome. One of these has ghost-like structures under electron microscopy. Catalase and thiolase are mislocated to the cytosol in these two groups. Another two groups are selectively defective for the import of specific peroxisome proteins: *Peb1* can import catalase but not thiolase; *Peb5* can import thiolase but not catalase. Both of these also show distinct clustering of peroxisomes inside the cell. These partial packaging defects may be related to the existence of multiple types of targeting signals in peroxisome proteins.

During the period this project was in progress, several laboratories had engaged in the study of peroxisome biogenesis using yeast as a model system. Kunau's group in Germany, which is the laboratory to have first isolated the peroxisome assembly mutants (*pas*) in *S. cerevisiae* (Erdmann et al., 1989), have reported the isolation of a total of 12 complementation groups of *pas* mutants

(Kunau et al., 1993). Tabak's group in Holland have reported the isolation of *pas* mutants using a variant of our procedure in which the catalase activity is inhibited with 3-amino-triazole (Van Der Leij et al., 1992), and more recently a different positive selection strategy based on using a chimeric gene encoding the bleomycin resistance protein linked to the peroxisomal protein luciferase (Elgersma et al., 1993). These two groups have compared their mutants genetically and totally 17 complementation groups have been reported. We have not yet had a chance to compare the *peb* mutants with all 17 *pas* groups but with the first 6 groups. The *peb2* is genetically equivalent to *pas1* as these two mutants can not complement with each other. The phenotype of *peb1* and *peb5* is similar but not identical to the *pas7* and *pas10* respectively.

Several groups have reported the isolation of mutants affecting peroxisome assembly in *Hansenula polymorpha* (Didion and Roggenkamp, 1990; Cregg et al., 1990; Waterham et al., 1992; Veenhuis et al., 1992; Titorenko et al., 1992) . At least a dozen complementation group have been isolated. Another yeast, *Pichia*

pastoris, has also been used by two groups to isolate mutants defective in peroxisome assembly independently (Gould et al., 1992; Liu et al., 1992) by screening for the failure of cells to grow on methanol. 11 complementation groups have ben isolated by these two groups (Subramani, 1993). Recently, Nuttley et al. (1993) have reported the isolation of mutants defective in peroxisome assembly from the yeast *Yarrowia lipolytica*. Several complementation groups have been identified. Even though it is not possible to genetically compare the mutants isolated from different species of the yeast, phenotype comparison and sequence analysis of the cloned genes (see below) indicate several mutant homologues have been identified in different yeasts (Van Der Leij et al., 1993; McCollum et al., 1993; Subramani, 1993).

Despite all this progress, the isolation of mutants deficient in peroxisome biogenesis seems not yet saturated. The machinery for peroxisome assembly and/or import is probably more complex than people have ever imagined. The selection strategy used in this project has been proved to be simple and efficient. More mutants

deficient in peroxisome biogenesis are expected to be identified with this method. The identified mutants can be readily used to clone the wild type yeast gene by functional complementation. The characterization of the gene products will lead to a fuller understanding of molecular events of peroxisome biogenesis in yeast cells. As the targeting signal, at best the SKL targeting signal, and probably the targeting signal of the thiolase, of peroxisomal proteins is very well conserved between yeast and mammalian cells, including human beings (Gould et al., 1990; Osumi et al., 1991; Swinkels et al., 1991; de Hoop et al., 1993). The import machinery is also likely to have been conserved. Therefore, the human and other mammalian homologs of the yeast PEB genes could also be cloned by transforming the mutants with human or other mammalian cDNA libraries constructed in a yeast expression vector (Schild et al., 1990). This, if successful, will help the elucidation of human peroxisome biogenesis directly.

In chapter 3, I described the in-depth characterization of *peb* mutants employing a new digitonin cell fractionation method. This

technique has been successfully used in mammalian cells (de Duve, 1965; Wanders et al., 1984). Here I demonstrated, the first time in the literature, that the yeast plasma membrane, peroxisome and mitochondria can be selectively permeabilized using different amounts of digitonin. This method avoids the mechanical breakage of the organelles and the attendant artefactual release of organelle proteins which is often seen in the classical fractionation procedure for peroxisome analysis. The five groups of *peb* mutants were analyzed with this new method for the packaging of three peroxisomal proteins, thiolase, catalase A and acyl-CoA oxidase. Each of these three proteins have different targeting information. Mutant *peb3* can package the three peroxisome proteins, but the steady state expression of catalase and thiolase is decreased when compared with wild type. The other four mutants have normal steady state protein expression, but are deficient in the import of all or some peroxisomal proteins. In the two mutants that do not have recognizable peroxisomes, these three proteins stay in the cytosol, consistent with the morphological studies described in Chapter 2. Mutant *peb1* packages catalase and AOx, but fails to

package thiolase. *Peb5* packages thiolase and AOx, but fails to package catalase. This results are consistent with, and extend, the morphological studies in Chapter 2. Based on these, I proposed that there are three pathways, or three branches of a pathway, involved in peroxisomal protein import, each being represented by one of the three peroxisome proteins examined. In this chapter, we also reported the first systematic tests of the quantitative property of chemiluminescence detection of immunoblotting. The observed linearity permits quantitative determination of antigens with chemiluminescent immuno-detection.

One potential use of the digitonin permeabilization is to establish an in vitro peroxisome import assay with semi-permeabilized cells in *S. cerevisiae*. An in vitro import system has been established in yeast using purified peroxisomes (Small et al., 1988; Thieringer et al., 1991). With this method, the import has been shown to be signal, temperature, and energy dependent. However, the fragility of peroxisomes in yeast cells, especially in *S. cerevisiae*, limited its wider application. The finding that digitonin

can selectively permeabilize the plasma membrane in yeast opens a new way for the in vitro study in peroxisome import. Actually, an in vitro import system using digitonin has been successfully used in mammalian cells for nuclear import (Adam et al., 1990). Wentland and Subramani (Wendland and Subramani, 1993) recently reported the establishment of an in vitro peroxisome import system in CHO cells using Streptolysin O, a compound with a similar property with digitonin. As was shown in Chapter 3, the disruption of plasma membrane at low digitonin concentrations is very gentle, with little disruption of peroxisomes. It is quite likely that organelles in such semi-permeabilized yeast cells will retain the competence to import peroxisomal proteins. These semi-intact cells can be used for peroxisome import studies. Peroxisomal proteins can be conjugated in vitro with fluorescent chemicals, such as fluorescein, for monitoring the import under the fluorescence microscope. The import efficiency using semi-permeabilized cells should be higher than that using purified peroxisomes as the organelles in the semi-intact cells are more close to their physiological state than the purified organelles. This procedure is simple and easy to operate as

there is no need to isolate the peroxisomes which is a very tedious process. This system should permit the identification of factors required for peroxisomal protein import in yeast. With the availability of abundant *peb* and *pas* mutants in *S. cerevisiae* and the cloned PEB gene and PAS genes, it is possible to combine this biochemical approach with the genetic approach to study the peroxisome biogenesis, which will facilitate the elucidation of the biochemical and molecular events of the import process.

In Chapter 4, the cloning of PEB1 and characterization of the gene product was presented. The PEB1 gene was cloned by functional complementation. The authenticity of the cloned PEB1 gene was confirmed by gene disruption and genetic experiments. Sequence analysis showed that PEB1 encodes a 42.32 kDa hydrophilic protein with six WD-40 repeats. It is repressed by glucose and derepressed by glycerol. However, oleate can not further induce its expression. Three copies of the hemagglutinin epitope tag were added to the C-terminal of the PEB1 gene for studying its subcellular location. It was demonstrated that the majority of Peb1p is peroxisomal

located. However, from the data I can not exclude the possibility that some of the Peb1p is located in the cytosol. The protease K digestion result suggests that the peroxisomal Peb1p likely is inside the peroxisome. Based on the phenotype of the *peb1* mutant and the localization of the Peb1p, I proposed that the Peb1p works as an intra-organelle receptor for thiolase import. It is envisioned that the thiolase first interacts with a receptor outside the peroxisome which then inserts the polypeptide through the peroxisome membrane. The Peb1p inside the peroxisome recognizes the inserted peptide and initiates a series of protein-protein interactions which result in the pulling of the protein into the organelle.

Recently, several yeast genes required for peroxisome biogenesis have been cloned by different laboratories in different species of yeast. Interesting, the majority of these yeast proteins have structurally homologs in the already identified proteins. Most of them can be classified into known protein families. The PAS1 gene was the first yeast gene identified as required for peroxisome assembly (Erdmann et al., 1991). It shares homology with SEC18p

(Eakle et al., 1988), NSF (Wilson et al., 1989) and CDC48 (Frohlich et al., 1991) in the two ATP binding domains. It is likely to be an ATPase. The gene product of the recently identified PAS8 in *S. cerevisiae* (Voorn-Brouwer et al., 1993) PAS5 gene in *P. pastoris* (Spong and Subramani, 1993) (presumably the PAS8 homolog) and PAY4 in *Y. lipolytica* (Nuttley et al., 1994) (also the homolog of PAS8) also contain an ATP binding domain which shares homology with that of Pas1p. They are presumably also ATPases. The phenotype of the corresponding mutants of these genes are similar: inability to package all the peroxisome matrix proteins. The function of these proteins in peroxisome assembly is yet to be addressed.

The Pas2p in *S. cerevisiae* showed striking homology to ubiquitin-conjugating enzymes UBC proteins in a highly conserved segment of about 140 residues including the essential "active site" cysteine residue (Wiebel and Kunau, 1992). Cell fractionation studies indicate that Pas2p associates with the peroxisome. As one well known function of the ubiquitin pathway is to mark substrate for subsequent degradation, a possible role of Pas2p could be to

mediate the degradation of a factor inhibiting peroxisome biogenesis through the ubiquitination of this factor.

The partial deduced amino acid sequences of Pas4p and Pas5p have been reported by Kunau (Kunau et al., 1993). Both proteins contain a cysteine-rich region near their C-termini which are related to the cysteine-rich regions of a new group of Zinc-Finger-like proteins, referred as type C_3HC_4 (Freemont et al., 1991). The PAF-1 identified in rodent (Tsukamoto et al., 1991) also has similar structure. The role of this motif in peroxisome assembly is unknown. Neither is the function of these two gene products known.

The recently identified Pas8 in *P. pastoris* (Causeret et al., 1993) and presumably its homolog in *S. cerevisiae* ,Pas10p (Van Der Leij et al., 1993), contain so called TPR repeats. Their possible role in peroxisome assembly has been discussed elsewhere in the thesis. The Pas3p, which has not been classified into any known family (Hohfeld et al., 1991), is a peroxisomal membrane protein. It probably functions during the peroxisome proliferation (Subramani, 1993).

The isolation of the PEB1 gene and the tagging of the gene product offers a new way to study the peroxisome protein import. The possible interaction of Peb1p with other components in the import machinery and with thiolase can be tested with the either biochemical approaches such as coimmunoprecipitation, or genetic approaches such as two-hybrid system.

The human disease RCDP has a similar biochemical defect to the *peb1* mutant: defective in thiolase packaging but not catalase packaging (Heikoop et al., 1990; Naidu et al., 1988). It would be extremely interesting to find out if the molecular defect of any RCDP cell lines is similar to that of *peb1* mutant. One way to study this would be to express the PEB1 gene in cells from RCDP patients and to test if the biochemical defect of RCDP cells can be restored. A positive result would facilitate the elucidation of the molecular basis of RCDP.

References

Adam, S. A., R. S. Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807-816.

Alexson, S. E. H., Y. Fujiki, H. Shio, and P. B. Lazarow. 1985. Partial disassembly of peroxisomes. *J. Cell Biol.* 101:294-304.

Alvares, K., A. Carrillo, P. M. Yuan, H. Kawano, R. I. Morimoto, and J. K. Reddy. 1990. Identification of cytosolic peroxisome proliferator binding protein as a member of the heat shock protein HSP70 family. *Proc. Natl. Acad. Sci. USA* 87:5293-5297.

Andre, P. J. and G. M. Small. 1993. Characterization of human peroxisome proliferation-activated receptor. *Mol. Biol. Cell* 4:106a.

Banroques, J. and J. N. Abelson. 1989. PRP4: a protein of th yeast U4/U6 small nuclear ribonucleoprotein particle. *Mol. Cell. Biol.* 9:3710-3719.

Baudhuin, P., H. Beaufay, Y. Rahman-Li, O. Z. Sellinger, R. Wattiaux, P. Jacques, and C. de Duve. 1964. Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. *Biochem. J.* 92:179-184.

Bellion, E. and J. M. Goodman. 1987. Proton ionophores prevent assembly of a peroxisomal protein. *Cell* 48:165-173.

Bergmeyer, H. U., M. Graβl, and H. -E. Walter. 1983a. Samples, reagents, assessment of results. In Methods of enzymatic analysis (volume II). H. U. Bergmeyer, J. Bergmeyer, and M. Graβl, editors. Verlag Chemie, Weiheim. 211-213.

Bergmeyer, H. U., M. Graβl, and H. -E. Walter. 1983b. Samples, reagents, assessment of results. In Methods of enzymatic analysis

(volume II). H. U. Bergmeyer, J. Bergmeyer, and M. Graβl, editors. Verlag Chemie, Weiheim. 189-191.

Bitter, G. A. and K. M. Egan. 1984. Expression of heterologus genes in *Saccharomyces cerevisiae* from vectors utilizing the glyceraldehyde-3-phosphate dehydrogenease gene promoter. *Gene* 32:263-274.

Blaauboer, B. J., C. W. van Holsteijn, R. Bleumink, W. C. Mennes, F. N. van Pelt, S. H. Yap, J. F. van Pelt, A. A. van Iersel, A. Timmerman, and B. P. Schmid. 1990. The effect of beclobric acid and clofibric acid on peroxisomal beta-oxidation and peroxisome proliferation in primary cultures of rat, monkey and human hepatocytes. *Biochem. Pharmacol.* 40:521-528.

Borst, P. 1989. Peroxisome biogenesis revisited. *Biochim. Biophys. Acta* 1008:1-13.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Causeret, C., M. Bentejac, and M. Bugaut. 1993. Proteins and enzymes of the peroxisomal membrane in mammals. *Biology of the Cell* 77:89-104.

Chance, B. 1950. The reactions of catalase in the presence of the notatin system. *Biochem. J.* 46:387-402.

Cohen, G., F. Fessl, A. Traczyk, J. Rytka, and H. Ruis. 1985. Isolation of the catalase A gene of *Saccharomyces cerevisiae* by complementation of the *cta1* mutation. *Mol. Gen. Genet.* 200:74-79.

Colbeau, A., J. Nachbaur, and P. M. Vignais. 1971. Enzymatic characterization and lipid composition of rat liver subcellular membranes. *Biochim. Biophys. Acta* 249:426-492.

Cooper, T. G. and H. Beevers. 1969. β Oxidation in glyoxysomes from

castor bean endosperm. *J. Biol. Chem.* 244:3514-3520.

Craig, E. A., J. Kramer, J. Shilling, M. Werner-Washberne, S. Holmes, J. Kosic-Smithers, and C. M. Nicolet. 1989. SSC1, an essential member of the yeast HSP70 multigene family, encodes a mitochondria protein. *Mol. Cell. Biol.* 9:3000-3008.

Cregg, J. M., I. J. Van Klei, G. J. Sulter, M. Veenhuis, and W. Harder. 1990. Peroxisome-deficient mutants of *Hanseluna polymorpha. Yeast* 6:87-97.

Cross, F. R. and A. H. Tinkelenberg. 1991. A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. *Cell* 65:875-883.

de Duve, C. 1965. The separation and characterization of subcellular particles. *Harvey Lect.* 59:49-87.

de Duve, C. 1967. General principles. In Enzyme Cytology. D. B. Roodyn, editor. Academic Press, New York. 1-26.

de Duve, C. and P. Baudhuin. 1966. Peroxisomes (microbodies and related particles). *Physiol. Rev.* 46:323-357.

de Hoop, M. J., W. L. Holtman, and G. AB. 1993. Human catalase is imported and assembled in peroxisomes of *Saccharomyces cerevisiae. Yeast* 9:59-69.

Diaz, R. and P. H. Stahl. 1989. Digitonin permeabilization procedures for the study of endosome acidification and function. In Methods in Cell Biology. A. M. Tartakoff, editor. Academic press, Inc., San Diego, California. 25-43.

Didion, T. and R. Roggenkamp. 1990. Deficiency of peroxisome assembly in a mutant of the methylotrophic yeast *Hansenula polymorpha. Curr. Genet.* 17:113-117.

Dmochowska, A., D. Dignard, R. Maleszka, and D. Y. Thomas. 1990.

Structure and transcriptional control of the *Saccharomyces cerevisiae* POX1 gene encoding acyl-coenzyme A oxidase. *Gene* 88:247-252.

Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* 16:6127-6145.

Duronio, R. J., J. I. Gordon, and M. S. Boguski. 1992. Comparative Analysis of the β transducin family with identification of several new members including PWP1, a nonessential gene of *Saccharomyces cerevisiae* that is divergently transcribed from NMT1. *Proteins: Structure, Function, and Genetics* 13:41-56.

Eakle, K., M. Bernstein, and S. D. Emr. 1988. Characterization of a component of yeast secretion machinery : identification of the SEC18 gene product. *Mol. Cell. Biol.* 8:4098-4109.

Einerhand, A. W., T. M. Voorn Brouwer, R. Erdmann, W. -H. Kunau, and H. F. Tabak. 1991. Regulation of transcription of the gene coding for peroxisomal 3- oxoacyl-CoA thiolase of Saccharomyces cerevisiae. *Eur. J. Biochem.* 200:113-122.

Elgersma, Y., M. Van Den Berg, H. F. Tabak, and B. Distel. 1993. An efficient positive selection procedure for the isolation of peroxisomal import and peroxisome assembly mutants of *Saccharomyces cerevisiae*. *Genetics* 135:731-740.

Elion, E. A., B. Sotterberg, and J. E. Kranz. 1993. FUS3 phosphorylates multiple components of the mating signal transduction cascade: evidence for STE12 and FAR1. *Mol. Biol. Cell* 4:495-510.

Erdmann, R., M. Veenhuis, D. Mertens, and W. -H. Kunau. 1989. Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 86:5419-5423.

Erdmann, R., F. F. Wiebel, A. Flessau, J. Rytka, A. Beyer, K-U. Frohlich, and W-H. Kunau. 1991. *PAS1*, a yeast gene required for peroxisome

biogenesis, encodes a member of a novel family of putative ATPases. *Cell* 64:499-510.

Fahimi, H. D. 1969. Cytochemical localization of peroxidatic activity of catalase in rat hepatic microbodies (peroxisomes). *J. Cell Biol.* 43:275-288.

Filipits, M., M. M. Simon, W. Rapatz, B. Hamilton, and H. Ruis. 1993. A *Saccharomyces cerevisiae* upstream activating sequence mediates induction of peroxisome proliferation by fatty acids. *Gene* 132:49-55.

Fong, H. K. W., J. B. Hurley, R. S. Hopkins, R. Miake-Lye, M. S. Johnson, R. F. Doolittle, and M. I. Simon. 1986. Repetitive segmental structure of the transducin β-subunit: homology with the CDC4 gene and identification of related mRNAs. *Proc. Natl. Acad. Sci. USA* 83:2162-2166.

Freemont, P. S., I. M. Hanson, and J. Trowsdale. 1991. A novel cysteine-rich sequence motif. *Cell* 64:483-484.

Frohlich, K. U., H. W. Fries, M. Rudiger, R. Erdmann, D. Botstein, and D. Mecke. 1991. Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. *J. Cell Biol.* 114:443-453.

Fujiki, Y., S. Fowler, H. Shio, A. L. Hubbard, and P. B. Lazarow. 1982. Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes. Comparison with endoplasmic reticulum and mitochondrial membranes. *J. Cell Biol.* 93:103-110.

Garrard, L. J. and J. M. Goodman. 1989. Two genes encode the major membrane-associated protein of methanol-induced peroxisomes from Candida boidinii. *J. Biol. Chem.* 264:13929-13937.

Gietl, C. 1990. Glyoxysomal malate dehydrogenase from watermelon is synthesized with an amino-terminal transit peptide. *Proc. Natl.*

Acad. Sci. USA 87:5773-5777.

Goebl, M. and M. Yanagida. 1991. The TPR snap helix: a novel protein repeat motif from mitosis to transcription. *Trends Biochem. Sci.* 16:173-176.

Goldfischer, S., C. L. Moore, A. B. Johnson, A. J. Spiro, M. P. Valsamis, H. K. Wisniewski, R. H. Ritch, W. T. Norton, I. Rapin, and L. M. Gartner. 1973. Peroxisomal and mitochondrial defects in the cerebro-hepato-renal syndrome. *Science* 182:62-64.

Gould, S. J., G-A. Keller, N. Hosken, J. Wilkinson, and S. Subramani. 1989. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* 108:1657-1664.

Gould, S. J., G-A. Keller, M. Schneider, S. H. Howell, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani. 1990. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. *EMBO J.* 9:85-90.

Gould, S. J., G-A. Keller, and S. Subramani. 1987. Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. *J. Cell Biol.* 105:2923-2931.

Gould, S. J., S. Krisans, G-A. Keller, and S. Subramani. 1990. Antibodies directed against the peroxisomal targeting signal of firefly luciferase recognize multiple mammalian peroxisomal proteins. *J. Cell Biol.* 110:27-34.

Gould, S. J., D. McCollum, A. P. Spong, J. A. Heyman, and S. Subramani. 1992. Development of the Yeast *Pichia pastoris* as a model organism for the genetic and molecular analysis of peroxisome assembly. *Yeast* 8:613-628.

Guarente, L. 1984. Yeast promoters: positive and negative elements. *Cell* 36:799-800.

Hajra, A. K. and J. E. Bishop. 1982. Glycerolipid biosynthesis in

peroxisomes via the acyl dihydroxyacetone phosphate pathway. *Ann. N. Y. Acad. Sci.* 386:170-182.

Harlow, E. and D. Lane. 1988. Antibodies. A laboratory manual. Cold Spring Harbor Laboratory, New York. 421 pp.

Heikoop, J. C., C. W. van Roermund, W. W. Just, R. Ofman, R. B. Schutgens, H. S. Heymans, R. J. Wanders, and J. M. Tager. 1990. Rhizomelic chondrodysplasia punctata. Deficiency of 3-oxoacyl-coenzyme A thiolase in peroxisomes and impaired processing of the enzyme. *J. Clin. Invest.* 86:126-130.

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* 28:351-359.

Hodge, V. J., S. J. Gould, S. Subramani, H. W. Moser, and S. K. Krisans. 1991. Normal cholesterol synthesis in human cells requires functional peroxisomes. *Biochem. Biophys. Res. Commun.* 181:537-541.

Hoffman, C. S. and F. Winston. 1987. A ten-minutes DNA preparation from yeast efficiently release autonomous plasmids for transformation of *Excherichia coli. Gene* 57:267-272.

Hohfeld, J., M. Veenhuis, and W. -H. Kunau. 1991. PAS3, a *Saccharomyces cerevisiae* gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. *J. Cell Biol.* 114:1167-1178.

Igual, J. C., E. Matallana, C. Gonzalez-Bosch, L. Franco, and J. E. Perez-Ortin. 1991. A new glucose-repressible gene identified from the analysis of chromatin structure in deletion mutants of yeast SUC2 locus. *Yeast* 7:379-389.

Imanaka, T., G. M. Small, and P. B. Lazarow. 1986. Import of acyl CoA oxidase into peroxisomes of rat liver in vitro: requirement of a protease-sensitive component(s) of the peroxisomal membrane. *J. Cell Biol.* 103:524a.

Imanaka, T., G. M. Small, and P. B. Lazarow. 1987. Translocation of acyl-CoA oxidase into peroxisomes requires ATP hydrolysis but not a membrane potential. *J. Cell Biol.* 105:2915-2922.

Issemann, I. and S. Green. 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347:645-650.

Ju, Q. and J. R. Warner. 1994. Ribosome synthesis during the growth cycle of *Saccharomyces cerevisiae*. *Yeast* 10:151-157.

Kamiryo, T., Y. Sakasegawa, and H. Tan. 1989. Expression and transport of *Candida tropicalis* peroxisomal acyl-CoA oxidase in the yeast *Candida maltosa*. *Agric. Biol. Chem.* 53:179-186.

Keller, G-A., S. Gould, M. DeLuca, and S. Subramani. 1987. Firefly luciferase is targeted to peroxisomes in mammalian cells. *Proc. Natl. Acad. Sci. USA* 84:3264-3268.

Keller, G-A., S. Krisans, S. J. Gould, J. M. Sommer, C. C. Wang, W. Schliebs, W. -H. Kunau, S. Brody, and S. Subramani. 1991. Evolutionary conservation of a microbody targeting signal that targets proteins to peroxisomes, glyoxysomes and glycosomes. *J. Cell Biol.* 114:893-904.

Keller, G-A., M. Pazirandeh, and S. Krisans. 1986. 3-Hydroxy-3-methylglutaryl coenzyme A reductase localization in rat liver peroxisomes and microsomes of control and cholestyramine-treated animals: quantitative biochemical and immunoelectron microscopical analyses. *J. Cell Biol.* 103:875-886.

Keller, H. and W. Wahli. 1993. Peroxisome proliferator-activated receptors: A link between endocrinology and nutrition? *Trends in Endocrinology and Metabolism* 4:291-296.

Kolodziej, P. A. and R. A. Young. 1991. Epitope tagging and protein surveillance. *Methods Enzymol.* 194:508-519.

Kragler, F., A. Langeder, J. Raupachova, M. Binder, and A. Hartig. 1993. Two independent peroxisomal targeting signals in catalase A of *Saccharomyces cerevisiae*. *J. Cell Biol.* 120:665-673.

Kunau, W. -H., A. Beyer, T. Franken, K. Götte, M. Marzioch, J. Saidowsky, A. Skaletz-Rorowski, and F. F. Wiebel. 1993. Two complementary approaches to study peroxisome biogenesis in *Saccharomyces cerevisiae*: Forward and reversed genetics. *Biochimie* 75:209-224.

Kunau, W. -H. and A. Hartig. 1992. Peroxisome biogenesis in *Saccharomyces cerevisiae*. *Antonie Van Leeuwenhoek* 62:63-78.

Kyte, J. and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105-132.

Lalwani, N. D., K. Alvares, M. K. Reddy, M. N. Reddy, I. Parikh, and J. K. Reddy. 1987. Peroxisome proliferator-binding protein: identification and partial characterization of nafenopin-, clofibric acid-, and ciprofibrate-binding proteins from rat liver. *Proc. Natl. Acad. Sci. USA* 84:5242-5246.

Lazarow, P. B. 1993. Genetic approaches to studying peroxisome biogenesis. *Trends in Cell Biology* 3:89-93.

Lazarow, P. B., V. Black, H. Shio, Y. Fujiki, A. K. Hajra, N. S. Datta, B. S. Bangaru, and J. Dancis. 1985. Zellweger syndrome: biochemical and morphological studies on two patients treated with clofibrate. *Pediatr. Res.* 19:1356-1364.

Lazarow, P. B. and C. de Duve. 1976. A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. *Proc. Natl. Acad. Sci. USA* 73:2043-2046.

Lazarow, P. B. and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489-530.

Lazarow, P. B. and H. W. Moser. 1989. Disorders of peroxisome biogenesis. In The Metabolic Basis of Genetic Disease. C. R. Scriver, A. L. . Beaudet, W. S. Sly, and A. D. Valle, editors. McGraw-Hill, New York. 1479-1509.

Lewin, A. S., V. Hines, and G. M. Small. 1990. Citrate synthase encoded by the CIT2 gene of *Saccharomyces cerevisiae* is peroxisomal. *Mol. Cell Biol.* 10:1399-1405.

Liu, H., X. Tan, M. Veenhuis, D. McCollum, and J. M. Cregg. 1992. An efficient screen for peroxisome-deficient mutant of *Pichia pastoris*. *J. Bacteriol.* 174:4943-4951.

Longley, R. P., A. H. Rose, and B. A. Knights. 1968. Composition of protoplast membrane from *Saccharomyces cerevisiae*. *Biochem. J.* 108:401-412.

McCammon, M. T., C. A. Dowds, K. Orth, C. R. Moomaw, C. A. Slaughter, and J. M. Goodman. 1990a. Sorting of peroxisomal membrane protein PMP47 from Candida boidinii into peroxisomal membranes of Saccharomyces cerevisiae. *J. Biol. Chem.* 265:20098-20105.

McCammon, M. T., M. Veenhuis, S. B. Trapp, and J. M. Goodman. 1990b. Association of glyoxylate and beta-oxidation enzymes with peroxisomes of *Saccharomyces cerevisiae*. *J. Bacteriol.* 172:5816-5827.

McCollum, D., E. Monosov, and S. Subramani. 1993. The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells--The PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is a member of the TPR protein family. *J. Cell Biol.* 121:761-774.

McConnell, S. J., L. C. Stewart, A. Talin, and M. P. Yaffe. 1990. Temperature-sensitive yeast mutants defective in mitochondrial inheritance. *J. Cell Biol.* 111:967-976.

Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty. 1986. Yeast

shuttle and integrative vectors with multiple cloning sites suitable for construction of lacZ fusions. *Gene* 45:299-310.

Naidu, S., G. Hoefler, P. A. Watkins, W. W. Chen, A. B. Moser, S. Hoefler, N. E. Rance, J. M. Powers, M. Beard, W. R. Green, and et-al. 1988. Neonatal seizures and retardation in a girl with biochemical features of X-linked adrenoleukodystrophy: a possible new peroxisomal disease entity. *Neurology* 38:1100-1107.

Nelson, J. and M. Wygant. 1992. Quantitational accuracy of film autoradiography: effects of 8-bit and 12-bit digitization. *J. NIH Research* 4:59-60.

Novikoff, A. B. and S. Goldfischer. 1969. Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. *J. Histochem. Cytochem.* 17:675-680.

Nuttley, W. M., A. M. Brade, G. A. Eitzen, M. Veenhuis, J. D. Aitchison, R. K. Szilard, J. R. Glover, and R. A. Rachubinski. 1994. PAY4, a gene required for peroxisome assembly in the yeast *Yarrowia lipolytica*, encodes a novel member of a family of putative ATPases. *J. Biol. Chem.* 269:556-566.

Nuttley, W. M., A. M. Brade, C. Gaillardin, G. A. Eitzen, J. R. Glover, J. D. Aitchison, and R. A. Rachubinski. 1993. Rapid identification and characterization of peroxisomal assembly mutants in *Yarrowia lipolytica*. *Yeast* 9:507-517.

Osmundsen, H., R. Z. Christiansen, and J. Bremer. 1980. Extramitochondrial shortening of long chain fatty acids and carnitine-dependent fatty acid oxidation. In Carnitine Biosynthesis, Metabolism, and Functions. R. A. Frenkel and J. D. McGarry, editors. Academic Press, Inc., New York. 127-142.

Osmundsen, H., C. E. Neat, and K. R. Norum. 1979. Peroxisomal oxidation of long chain fatty acids. *FEBS Lett.* 99:292-296.

Osumi, T. and T. Hashimoto. 1978. Acyl-CoA oxidase of rat liver: a

new enzyme for fatty acid oxidation. *Biochem. Biophys. Res. Commun.* 83:479-485.

Osumi, T., T. Tsukamoto, S. Hata, S. Yokota, S. Miura, Y. Fujiki, M. Hijikata, S. Miyazawa, and T. Hashimoto. 1991. Amino-terminal presequence of the precursor of peroxisomal 3-ketoacyl-CoA thiolase is a cleavable signal peptide for peroxisomal targeting. *Biochem. Biophys. Res. Commun.* 181:947-954.

Peterson, T. A., J. Yochem, B. Byers, M. F. Nunn, P. H. Duesberg, R. F. Doolittle, and S. I. Reed. 1984. A relationship between the yeast cell cycle genes CDC4 and CDC36 and ets sequence of oncogenic virus E26. *Nature* 309:556-558.

Pringle, J. R., A. M. Amans, D. J. Drubin, and B. K. Haarer. 1991. Immunofluorescence methods for yeast. In Guide to yeast genetics and molecular biology. C. Guthrie and G. Fink, editors. Academic press, Inc., San Diego. 565-601.

Prydz, K., B. F. Kase, I. Bjorkhem, and J. I. Pedersen. 1986. Formation of chenodeoxycholic acid from 3 alpha, 7 alpha-dihydroxy-5 beta-cholestanoic acid by rat liver peroxisomes. *J. Lipid Res.* 27:622-628.

Purdue, P. E., Y. Takada, and C. J. Danpure. 1990. Identification of mutations associated with peroxisome-to-mitochondrion mistargeting of alanine/glyoxylate aminotransferase in primary hyperoxaluria type 1. *J. Cell Biol.* 111:2341-2351.

Rattray, J. B. M., A. Schibeci, and B. K. Kidby. 1975. Lipids of yeast. *Bacteriological reviews* 39(3):197-231.

Rhodin, J. 1954. Correlation of ultrastructural organization and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney. Karolinska Institutet, 1 pp.

Rodricks, J. V. and D. Turnbull. 1987. Interspecies differences in peroxisomes and peroxisome proliferation. *Toxicol. Ind. Health*

3:197-212.

Roggenkamp, R., H. Sahm, W. Hinkelmann, and F. Wagner. 1975. Alcohol oxidase and catalase in peroxisomes of methanol-grown Candida boidinii. *Eur. J. Biochem.* 59:231-236.

Roggieri, R., K. Tanaka, M. Nakafuku, Y. kaziro, A. Toh-e, and K. Matsumoto. 1989. MSI1, a negative regulator of the RAS-cAMP pathway in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* 86:8778-8782.

Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink. 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* 60:237-243.

Rothstein, R. 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* 194:281-301.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor, New York.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.

Santos, M. J., S. Hoefler, A. B. Moser, H. W. Moser, and P. B. Lazarow. 1992. Peroxisome assembly mutations in humans: structural heterogeneity in Zellweger syndrome. *J. Cell. Physiol.* 151:103-112.

Santos, M. J., T. Imanaka, H. Shio, G. M. Small, and P. B. Lazarow. 1988. Peroxisomal membrane ghosts in Zellweger syndrome-aberrant organelle assembly. *Science* 239:1536-1538.

Schild, D., A. J. Brake, M. C. Kiefer, D. Young, and P. J. Barr. 1990. Cloning of three human multifunctional *de novo* purine biosynthetic genes by functional complementation of yeast mutations. *Proc. Natl. Acad. Sci. USA* 87:2916-2920.

Schultz, L. D. and J. D. Friesen. 1983. Nucleotide sequence of the *tcm1* gene (ribosomal protein L3) of *Saccharomyces cerevisiae*. *J. Bacteriol.* 155:8-14.

Sherman, F., G. R. Fink, and J. B. Hicks. 1986. Laboratory Course Manual for Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor. 1 pp.

Shimozawa, N., Y. Suzuki, T. Orii, A. Moser, H. W. Moser, and R. J. A. Wanders. 1993. Standardization of complementation grouping of peroxisome-deficient disorders and the second Zellweger patient with peroxisomal assembly factor-1 (PAF-1) defect. *Am. J. Hum. Genet.* 52:843-844.

Shimozawa, N., T. Tsukamoto, Y. Suzuki, T. Orii, Y. Shirayoshi, T. Mori, and Y. Fujiki. 1992. A human gene responsible for Zellweger syndrome that affects peroxisome assembly. *Science* 255:1132-1134.

Sikorski, R. S. and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122:19-27.

Skoneczny, M., A. Chelstowska, and J. Rytka. 1988. Study of the coinduction by fatty acids of catalase A and acyl-CoA oxidase in standard and mutant *Saccharomyces cerevisiae* strains. *Eur. J. Biochem.* 174:297-302.

Slot, J. W. and H. J. Geuze. 1984. Gold markers for single and double immunolabelling of ultrathin cryosections. In Immunolabelling for electron microscopy. J. M. Polark and J. M. Vandell, editors. Elsevier Sci Publ, Amersterdam. 129-142.

Small, G. M. 1993. Peroxisome biogenesis. In Peroxisomes: Biology and Importance in Toxicology and Medicine. G. Gibson and B. Lake, editors. Taylor & Francis, Ltd., Washington, D.C.. 1-18.

Small, G. M., T. Imanaka, H. Shio, and P. B. Lazarow. 1987. Efficient association of *in vitro* translation products with purified, stable *Candida tropicalis* peroxisomes. *Mol. Cell Biol.* 7:1848-1855.

Small, G. M. and P. B. Lazarow. 1987. Import of the carboxyterminal portion of acyl-CoA oxidase into peroxisomes of *Candida tropicalis*. *J. Cell Biol.* 105:247-250.

Small, G. M., L. J. Szabo, and P. B. Lazarow. 1988. Acyl-CoA oxidase contains two targeting sequences each of which can mediate protein import into peroxisomes. *EMBO J.* 7:1167-1173.

Sohaskey, M., K. Campbell, and J. M. Goodman. 1993. Further evidence for involvement of a proton-motive force in import of proteins into peroxisomes. *Mol. Biol. Cell* 4:107a.

Spong, A. P. and S. Subramani. 1993. Cloning and characterization of *PAS5*: A gene required for peroxisome biogenesis in the methylotrophic yeast *Pichia pastoris*. *J. Cell Biol.* 123:535-548.

Stamellos, K. D., J. E. Shackelford, R. D. Tanaka, and S. K. Krisans. 1992. Mevalonate kinase is localized in rat liver peroxisomes. *J. Biol. Chem.* 267:5560-5568.

Stevens, B. J. 1977. Variation in number and volume of the mitochondria in yeast according to growth conditions. A study based on serial sectioning and computer graphic reconstruction. *Biol. Cellulaire.* 28:37-56.

Stoeckle, M. Y. and J. Guan. 1993. Improved resolution and sensitivity of Northern blots using polyacrylamide-urea gels. *Biotechniques* 15:227-231.

Stuart, R. A,, D. M. Cyr, E. A. Craig, and W. Neupert. 1994. Mitochondrial molecular chaperones: their role in protein translocation. *Trends Biochem. Sci.* 19:87-92.

Subramani, S. 1993. Protein import into peroxisomes and biogenesis

of the organelle. *Annu. Rev. Cell Biol.* 9:445-478.

Suzuki, Y., N. Shimozawa, T. Orii, N. Igarashi, N. Kono, A. Matsui, Y. Inoue, S. Yokota, and T. Hashimoto. 1988. Zellweger-like syndrome with detectable hepatic peroxisomes: a variant form of peroxisomal disorder. *J. Pediatr.* 113:841-845.

Suzuki, Y., N. Shimozawa, S. Yajima, T. Orii, S. Yokota, Y. Tashiro, T. Osumi, and T. Hashimoto. 1992. Different intracellular localization of peroxisomal proteins in fibroblasts from patients with aberrant peroxisome assembly. *Cell Struct. Funct.* 17:1-8.

Swinkels, B. W., S. J. Gould, A. G. Bodnar, R. A. Rachubinski, and S. Subramani. 1991. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO J.* 10:3255-3262.

Thieringer, R., H. Shio, Y. Han, G. Cohen, and P. B. Lazarow. 1991. Peroxisomes in *Saccharomyces cerevisiae*: Immunofluorescence analysis and import of catalase A into isolated peroxisomes. *Mol. Cell Biol.* 11:510-522.

Titorenko, V. I., H. R. Waterham, P. Haima, W. Harder, and M. Veenhuis. 1992. Peroxisome biogenesis in Hansenula polymorpha: different mutations in genes, essential for peroxisome biogenesis, cause different peroxisomal mutant phenotypes. *FEMS. Microbiol. Lett.* 74:143-148.

Tsukamoto, T., S. Miura, and Y. Fujiki. 1991. Restoration by a 35K membrane protein of peroxisome assembly in a peroxisome-deficient mammalian cell mutant. *Nature* 350:77-81.

Tsukamoto, T., S. Yokota, and Y. Fujiki. 1990. Isolation and characterization of Chinese hamster ovary cell mutants defective in assembly of peroxisomes. *J. Cell Biol.* 110:651-660.

Tsuneoka, M., A. Yamamoto, Y. Fujiki, and Y. Tashiro. 1988. Nonspecific lipid transfer protein (sterol carrier protein-2) is

located in rat liver peroxisomes. *J. Biochem.* 104:560-564.

Tuite, M. F. 1989. Protein synthesis. In The Yeasts. Volume 3: Metabolism and Physiology of Yeasts. A. H. Rose and J. S. Harrison, editors. Academic Press, San Diego, CA. 161-204.

Tyers, M., G. Tokiwa, R. Nash, and B. Futcher. 1992. The Cln3-Cdc28 kinase complex of S. cerevisiae is regulated by proteolysis and phosphorylation. *EMBO J.* 11:1773-1784.

Van den Bosch, H., R. B. H. Schutgens, R. J. A. Wanders, and J. M. Tager. 1992. Biochemistry of peroxisomes. *Annu. Rev. Biochem.* 61:157-197.

Van Der Leij, I., M. M. Franse, Y. Elgersma, B. Distel, and H. F. Tabak. 1993. PAS10 is a tetratricopeptide-repeat protein that is essential for the import of most matrix proteins into peroxisomes of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* 90:11782-11786.

Van Der Leij, I., M. Van Den Berg, R. Boot, M. Franse, B. Distel, and H. F. Tabak. 1992. Isolation of peroxisome assembly mutants from *Saccharomyces cerevisiae* with different morphologies using a novel positive selection procedure. *J. Cell Biol.* 119:153-162.

van der Voorn, L. and H. L. Ploegh. 1992. The WD-40 repeat. *FEBS Lett.* 307:131-134.

van Dijken, J. P., M. Veenhuis, C. A. Vermeulen, and W. Harder. 1975. Cytochemical localization of catalase activity in methanol-grown *Hansenula polymorpha. Arch. Microbiol.* 105:261-267.

Van Tuinen, E. and H. Riezman. 1987. Immunolocalization of glyceral dehyde-3-phosphate dehydrogenase, hexokinase and carboxypeptidase Y in yeast cells at ultrastructural level. *J. Histochem. Cytochem.* 35:327-333.

Veenhuis, M., M. Mateblowski, W. -H. Kunau, and W. Harder. 1987. Proliferation of microbodies in *Saccharomyces cerevisiae. Yeast*

3:77-84.

Veenhuis, M., I. J. van der Klei, V. Titorenko, and W. Harder. 1992. *Hansenula polymorpha*: An attractive model organism for molecular studies of peroxisome biogenesis and function. *FEMS Microbiol. Lett.* 100:393-404.

Voorn-Brouwer, T., I. Van Der Leij, W. Hemrika, B. Distel, and H. F. Tabak. 1993. Sequence of the *PAS8* gene, the product of which is essential for biogenesis of peroxisomes in *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta: Gene Structure and Expression* 1216:325-328.

Wanders, R. J. A., M. Kos, B. Roest, A. J. Meijer, G. Schrakamp, H. S. A. Heymans, W. H. H. Tagelaers, H. van der Bosch, R. B. H. Schutgens, and J. M. Tager. 1984. Activity of peroxisomal enzymes and intracellular distribution of catalase in Zellweger syndrome. *Biochem. Biophys. Res. Commun.* 123:1054-1061.

Wanders, R. J. A., R. B. H. Schutgens, and J. M. Tager. 1985. Peroxisomal matrix enzymes in Zellweger syndrome: Activity and subcellular localization in liver. *J. Inherited Metab. Dis.* 8 Suppl. 2:151-152.

Wang, T. W., A. S. Lewin, and G. M. Small. 1992. A negative regulating element controlling transcription of the gene encoding acyl-CoA oxidase in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 20:3495-3500.

Waterham, H. R., V. I. Titorenko, I. J. van der Klei, W. Harder, and M. Veenhuis. 1992. Isolation and characterization of peroxisomal protein import (Pim-) mutants of *Hansenula polymorpha*. *Yeast* 8:961-972.

Wendland, M. and S. Subramani. 1993. Cytosol-dependent peroxisomal protein import in a permeabilized cell system. *J. Cell Biol.* 120:675-685.

Whiteway, M., L. Hougan, D. Dignard, D. Y. Thomas, L. Bell, G. C. Saari, F. J. Grant, P. O'Hara, and V. L. MacKay. 1989. The STE4 and the STE18 genes of yeast encode potential β and γ sununits of the mating factor receptor-coupled G protein. *Cell* 56:467-477.

Wiebel, F. F. and W. -H. Kunau. 1992. The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin-conjugating enzymes. *Nature* 359:73-78.

Wiemer, E., S. Brul, W. W. Just, M. Van Den Berg, P. J. Weijers, R. B. H. Schutgens, H. Van den Bosch, A. Schram, R. J. A. Wanders, and J. M. Tager. 1989. Presence of peroxisomal membrane proteins in liver and fibroblasts from patients with Zellweger syndrome and related disorders: Evidence for the existence of ghosts. *Eur. J. Cell Biol.* 50:407-417.

Williams, F. E. and R. J. Trumbly. 1990. Characterization of TUP1, a mediator of glucose repression in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 10:6500-6511.

Williams, F. E., U. Varanasi, and R. J. Trumbly. 1991. The CYC8 and TUP1 proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. *Mol. Cell. Biol.* 11:3307-3316.

Wilson, D. W., C. A. Wilcox, G. C. Flynn, C. Ellson, W. -J. Kuang, W. J. Henzel, M. R. Block, A. Ullrich, and J. E. Rothman. 1989. A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* 339:355-359.

Wilson, I. A., H. L. Niman, R. A. Houghten, A. R. Cherenson, M. C. Connolly, and R. A. Lerner. 1984. The structure of an antigenic determinant in a protein. *Cell* 37:767-778.

Wykle, R. L. 1977. Brain. In Lipid Metabolism in Mammals. F. Snyder, editor. Plenum Press, New York. 317-366.

Yajima, S., Y. Suzuki, N. Shimozawa, S. Yamaguchi, T. Orii, Y. Fujiki, T.

Osumi, T. Hashimoto, and H. W. Moser. 1992. Complementation study of peroxisome-deficient disorders by immunofluorescence staining and characterization of fused cells. *Hum. Genet.* 88:491-499.

Zoeller, R. A. and C. R. H. Raetz. 1986. Isolation of animal cell mutants deficient in plasmalogen biosynthesis and peroxisome assembly. *Proc. Natl. Acad. Sci. USA* 83:5170-5174.