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Identification and Characterization of *Yl*Pex5p, a Component of the Peroxisomal Translocation Machinery of *Yarrowia lipolytica*

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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24 January 2006
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ABSTRACT

Peroxisomes are ubiquitous eukaryotic organelles. *PEX* genes encode peroxins (proteins required for peroxisome assembly). A genetic screen identified mutants of the yeast *Yarrowia lipolytica* that fail to assemble functional peroxisomes. One strain, *pex5-1*, has abnormally small, clustered peroxisomes that are often surrounded by membranous material. The *PEX5* gene encodes *Yl*Pex5p. Pex5 proteins have been shown to function as receptors for type 1 peroxisomal targeting signals (PTS1s) in other species, interacting with PTS1s through their characteristic tetratricopeptide (TPR) domain. Biochemical characterization of the mutants *pex5-1* and *pex5-KO* (a *PEX5* gene disruption strain) showed that *Yl*Pex5p is a component of the peroxisomal translocation machinery. Mutations in *PEX5* prevent the translocation into the matrix of most proteins successfully targeted to peroxisomes. These proteins, including a 62-kD polypeptide (p62) recognized by antibodies to a PTS1 (SKL), seem to be trapped in the peroxisomal membrane at an intermediate stage of translocation in *pex5* mutants. The evidence presented suggests that there are at least two distinct translocation machineries for peroxisomal protein import. *Yl*Pex5p is intraperoxisomal. In wild-type peroxisomes, *Yl*Pex5p is associated primarily with the inner surface of the peroxisomal membrane, but approximately one-third of *Yl*Pex5p is localized to the peroxisomal matrix. Most *Yl*Pex5p in the matrix is complexed with two anti-SKL-reactive polypeptides: p62, and a 64-kD protein (p64). However, in *pex5-1* cells, peroxisomal Pex5 protein is localized exclusively to the matrix and forms no complex with the membrane-associated p62. The nature of the association of *Yl*Pex5p with PTS1 signals was investigated with *in vitro* binding assays. A recombinant *Yl*Pex5p fusion protein interacted specifically,

directly and autonomously with a protein terminating in a PTS1. *In vitro* translated wild-type *Yl*Pex5p specifically recognized functional PTS1s. This activity is abrogated by a glycine-to-aspartic acid substitution at a conserved residue in the TPR domain (G455D) of the protein encoded by the *pex5-1* allele. Deletion analysis demonstrated that an intact TPR domain of *Yl*Pex5p is necessary, but not sufficient, for both interaction with a PTS1 and functional complementation of the *pex5-KO* strain. A model for *Yl*Pex5p action is presented.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

20KgP	pellet resulting from a centrifugation at 20,000 x *g*
20KgS	supernatant resulting from a centrifugation at 20,000 x *g*
A	Ampere
AAA	ATPases associated with diverse cellular activities
ADP	adenosine diphosphate
ARF	ADP-ribosylation factor
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pair
BSA	bovine serum albumin
c	centi ($x10^{-2}$)
C.	*Candida*
C-	carboxyl-
°C	degrees Celcius
Cb	*Candida boidinii*
CG	complemetation group of human peroxisome biogenesis disorders
CHO	Chinese hamster ovary
Ci	Curie
CoA	coenzyme A
cpm	counts per minute
d	day
D	Dalton
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
E.	*Escherichia*
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
g	gram
g	gravitational force
GST	glutathione-S-transferase
h	hour
H.	*Hansenula*
Hp	*Hansenula polymorpha*
HRP	horseradish peroxidase
IgG	immunoglobulin G
IPTG	isopropyl β-D-thiogalactoside
IRD	infantile Refsum disease
J	Joule
k	kilo ($x10^{3}$)
L	litre
λ	wavelength

m	metre *or* milli ($x10^{-3}$)
M	moles per litre
MBP	maltose binding protein
MFE	multifunctional β-oxidation enzyme
min	minute
μ	micro ($x10^{-6}$)
n	nano ($x10^{-9}$)
N-	amino-
NALD	neonatal adrenoleukodystrophy
NEM	N-ethylmaleimide
$OD_{\#}$	optical density at a wavelength of # nm
ORF	open reading frame
P.	*Pichia*
p62	62-kD anti-SKL-reactive polypeptide
p64	64-kD anti-SKL-reactive polypeptide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEX#	wild-type gene encoding Pex#p
pex#	non-functional *PEX#* gene
Pex#p	peroxin # protein
pH	$-\log[H^+]$
PMP	peroxisomal membrane protein
PNS	post-nuclear supernatant
Pp	*Pichia pastoris*
PTS	peroxisomal targeting signal
RCDP	rhizomelic chondrodysplasia punctata
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
S.	*Saccharomyces*
Sc	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
sec	second
SH3	Src homology-3
TCA	trichloroacetic acid
TPR	tetratricopeptide repeat
U	unit of enzyme activity
v	volume
V	Volt
w	weight
Y.	*Yarrowia*
Yl	*Yarrowia lipolytica*
ZS	Zellweger syndrome

CHAPTER 1

Introduction

1.1 Principles of organelle biogenesis

Eukaryotic cells possess a system of membrane-bound organelles (the endoplasmic reticulum (ER) and secretory pathway organelles, mitochondria, the nucleus, peroxisomes, vacuoles/lysosomes and, in plants, chloroplasts) in which specific processes are housed. To achieve this compartmentalization of function, eukaryotic organisms must possess systems so that the particular proteins, nucleic acids and lipids that each organelle requires are correctly assembled into a functional unit. A nuclear-encoded protein destined for organellar import is generally directed to the appropriate compartment by a specific *cis*-acting targeting signal that is recognized by a receptor which is located at, or shuttles proteins to, the organelle. Once it has reached the organelle, the protein is either translocated across, or integrated into, the membrane (Schatz and Dobberstein, 1996). Membrane lipids must be distributed from their sites of synthesis to the different cellular membranes as required (Vance and Shiao, 1996). There must also be mechanisms in place to ensure that daughter cells inherit, or can regenerate, all cellular compartments (Warren and Wickner, 1996). Additionally, cells must be able to regulate the number and composition of organelles to meet varying cellular needs (Nunnari and Walter, 1996). This chapter will focus on some of the molecular processes involved in peroxisome biogenesis.

1.2 Peroxisomes and their functions

Peroxisomes, along with glyoxysomes of plants and glycosomes of trypanosomes, are members of the microbody family of organelles, a class of organelles that is found in most eukaryotic organisms. Peroxisomes are 0.1-1.0 μm in diameter, are bounded by a single unit

membrane, have an electron-dense, granular matrix and, in some species, have a paracrystalline core (Lazarow and Fujiki, 1985). In electron micrographs, peroxisomes appear roughly circular, suggesting that the organelles are essentially spherical. However, evidence for a peroxisomal reticulum comes from electron microscopical analysis of serial sections of cells from rat and mouse liver and mouse sebaceous glands (Gorgas, 1984; 1985; Yamamoto and Fahimi, 1987). Peroxisomes have been believed for many years to arise by growth and fission of pre-existing organelles (Lazarow and Fujiki, 1985), implying that at least one peroxisome must be segregated to daughter cells during cell division. However, there is also recent evidence for *de novo* peroxisome formation and for involvement of the ER as a source of membranes for peroxisome assembly (reviewed in Erdmann *et al.*, 1997; Kunau and Erdmann, 1988; Titorenko and Rachubinski, 1998a).

Peroxisomes have many diverse functions, depending on the organism, cell type, developmental stage and cellular environment (reviewed in Borst, 1989; van den Bosch *et al.*, 1992; Subramani, 1993). Peroxisomes were originally defined by de Duve as cytoplasmic organelles containing at least one hydrogen peroxide-producing oxidase and catalase to decompose the hydrogen peroxide (de Duve and Baudhuin, 1966), although this strict definition does not apply to all microbodies (Subramani, 1993). The oxidation of many compounds occurs in these organelles, but the most notable function of peroxisomes is the β-oxidation of fatty acids, resulting in the production of acetyl-CoA (Figure 1-1). Depending on the organism, other functions of peroxisomes can include reactions involved in the synthesis of plasmalogens, cholesterol and bile acids, purine and amino acid catabolism, the utilization of alcohols, photorespiration, and penicillin biosynthesis (Tolbert, 1981; van den

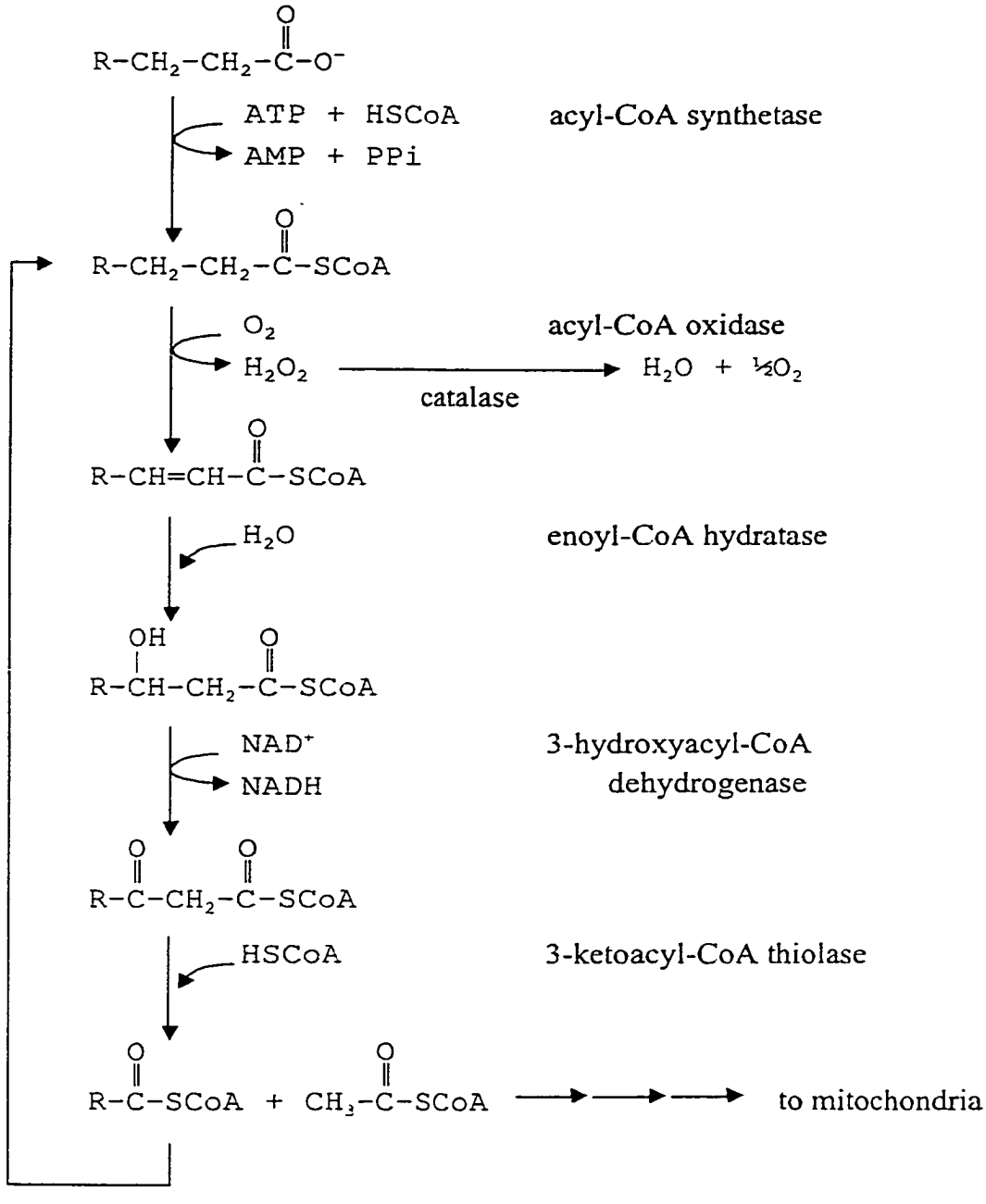

Figure 1-1. Peroxisomal β-oxidation.

Bosch *et al.*, 1992; Subramani, 1993). Glyoxysomes and glycosomes are often considered to be specialized peroxisomes, harbouring β-oxidation systems as well as enzymes of the glyoxylate cycle or glycolytic pathway, as their names imply (van den Bosch *et al.*, 1992).

Cells can vary the number and enzyme content of peroxisomes in response to metabolic or developmental changes. For example, yeasts growing on glucose-containing media contain one or a few small peroxisomes per cell. Transferring the cells to media with a carbon source that requires peroxisomes for its metabolism, such as oleic acid (*Candida albicans*, *Candida boidinii*, *Candida tropcalis*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Pichia pastoris*) or methanol (*Hansenula polymorpha*, *C. boidinii*, *P. pastoris*) results in a rapid increase in the size and number of peroxisomes, and is accompanied by an increase in the synthesis of peroxisomal proteins (Veenhuis and Harder, 1987; van der Klei and Veenhuis, 1997). A similar change is seen in the livers of rodents upon the administration of a group of naturally occurring or synthetic compounds collectively known as peroxisome proliferators, which includes certain hypolipidemic drugs, plasticizers, fatty acid analogues, herbicides, industrial solvents and anti-inflammatory drugs (Reddy and Chu, 1996). In plants, developmental cues alter peroxisome function. The "glyoxysomes" of germinating oil-bearing seedlings (containing an active β-oxidation system and glyoxylate cycle) are converted to "peroxisomes" (specialized for photosynthetic glycolate catabolism) following the greening of leaves (Tolbert, 1981; Olsen and Harada, 1995).

1.3 Peroxisomes and human disease

The importance of peroxisomes for humans is evidenced by a number of serious

disease states arising from peroxisome dysfunction (reviewed in Lazarow and Moser, 1995; Moser and Moser, 1996; Wanders, 1999). The first class of diseases arises from a defect in peroxisome biogenesis; peroxisomes are undetectable or morphologically abnormal, and there are deficiencies in multiple peroxisomal functions. Various peroxisomal enzymes are mislocalized to the cytosol or are absent (Wanders *et al.*, 1985; Schram *et al.*, 1986), but membrane proteins are often correctly assembled into peroxisomal "ghosts", which equilibrate at an unusually light density during isopycnic centrifugation (Santos *et al.*, 1988a; Santos *et al.*, 1988b; Gärtner *et al.*, 1991). This class of diseases includes classical Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and hyperpipecolic acidemia. ZS, NALD and IRD patients share a similar range of clinical manifestations, including an accumulation of very long chain fatty acids ($\geq C_{24}$, which cannot be broken down by the mitochondrial β-oxidation pathway that is also present in animal cells), plasmalogen deficiency, severe mental retardation, hypotonia, neuronal migration defects, hepatic dysfunction and retinopathy. These three diseases are considered to be part of a continuum, with ZS being the most severe (death usually occurs within the first year) and IRD the least severe (death usually by age 10). Rhizomelic chondrodysplasia punctata type 1 (RCDP-1) is a variant of this class of diseases, in which the import of only a few matrix proteins is affected, and there are fewer biochemical abnormalities. The disease is still quite severe, however, with death usually occurring within the first few years. Symptoms of RCDP-1 include defective plasmalogen synthesis, shortened limbs, epiphyseal calcific stippling, mental retardation and cataracts. There are at least thirteen complementation groups for the peroxisome biogenesis disorders, with twelve identified for the ZS spectrum and one for

RCDP-1 (Wanders, 1999); the genetic bases for ten of these are known (see Table 1-1). Disorders of peroxisome biogenesis have an autosomal recessive inheritance and occur at a combined frequency of 1 in 25,000 to 1 in 50,000 live births (Lazarow and Moser, 1995).

The second class of peroxisomal disorders results from a deficiency in the activity of a single protein or enzyme, but where the peroxisomes are otherwise intact. Examples of this class include X-linked adrenoleukodystrophy (deficiency in a protein of the ATP binding cassette superfamily of membrane transporters), primary hyperoxaluria type 1 (alanine:glyoxylate aminotransferase deficiency), classical Refsum disease (phytanoyl-CoA hydroxylase deficiency), RCDP-2 and -3 (dihydroxyacetonephosphate acyltransferase and alkyl-dihydroxyacetonephosphate synthetase deficiency, respectively) as well as deficiencies in individual β-oxidation enzymes. Each disease has a different subset of clinical manifestations that are also common to the peroxisome biogenesis disorders (Lazarow and Moser, 1995; Moser and Moser, 1996; Wanders, 1999).

1.4 Import of proteins into peroxisomes

Peroxisomes do not contain DNA (Kamiryo *et al.*, 1982). Peroxisomal proteins are encoded by the nuclear genome, are translated almost exclusively on free polysomes in the cytosol, and are imported post-translationally into the organelle, usually without detectable modification (reviewed in Lazarow and Fujiki, 1985). Therefore, the information that directs proteins to peroxisomes is generally contained within the sequence of the mature protein.

1.4.1 Targeting signals for matrix proteins

To date, three types of peroxisomal targeting signal (PTS) have been identified (reviewed in Subramani, 1998; Hettema *et al.*, 1999). PTS1, the most commonly used signal, is a conserved tripeptide motif found at the extreme C-terminus of many peroxisomal proteins. The canonical PTS1 sequence is SKL, which was first identified in firefly luciferase, and was found to be necessary and sufficient to direct proteins to peroxisomes in monkey kidney CV-1 cells (Gould *et al.*, 1989). The fact that luciferase was also targeted to peroxisomes in plant and yeast cells (Gould *et al.*, 1990a), as well as the observation that antiserum directed against the SKL motif reacted specifically with proteins from peroxisomes, glyoxysomes and glycosomes of various species (Gould *et al.*, 1990b; Keller *et al.*, 1991), first suggested the universality of this targeting mechanism. Several conservative substitutions in the tripeptide are tolerated with respect to luciferase targeting in CV-1 cells (Gould *et al.*, 1989). From this, a general consensus for the PTS1 emerged, which is (S/A/C)-(K/R/H)-(L), or a small uncharged residue in the first position, a basic residue in the middle, and a large hydrophobic residue in the terminal position. Further research has demonstrated that the range of acceptable C-terminal tripeptides is much expanded, but that allowable deviation from the consensus is both species- and context-specific (Aitchison *et al.*, 1991; Blattner *et al.*, 1992; Motley *et al.*, 1995; Elgersma *et al.*, 1996b; Purdue and Lazarow, 1996; Mullen *et al.*, 1997; Lametschwandtner *et al.*, 1998), with the amino acid residues immediately preceding the C-terminal tripeptide being the most critical, especially if it is an atypical signal (Purdue and Lazarow, 1996; Mullen *et al.*, 1997; Lametschwandtner *et al.*, 1998). The Pex5 family of peroxins are PTS1 receptors (discussed in Section 1.6). A comparison of the binding properties of *S. cerevisiae* and human Pex5p reveal that there is

species specificity in their preferences for both C-terminal tripeptides, as well as for adjacent sequences (Lametschwandtner *et al.*, 1998)

PTS2 is a much less commonly used targeting signal, but it is also evolutionarily conserved. PTS2 sequences are found most notably in peroxisomal thiolases from yeast to humans and in a few other proteins (see de Hoop and AB, 1992; Subramani, 1998 for reviews). This sequence is located near the N-terminus and has the consensus structure (R/K)-(L/V/I)-$(X)_5$-(H/Q)-(L/A). Unlike PTS1s, which are part of the mature proteins on which they are found, PTS2s are in some cases part of cleavable prepieces (Hijikata *et al.*, 1987; 1990; Bodnar and Rachubinski, 1990; Gietl, 1990; Osumi *et al.*, 1991; Swinkels *et al.*, 1991; Gietl *et al.*, 1994) that are normally removed during or after import into the peroxisomal matrix, although signal cleavage is not mechanistically coupled to import (Gietl *et al.*, 1994; Motley *et al.*, 1994). Also unlike PTS1 signals, which cannot function from internal locations (even one amino acid residue appended to the C-terminus of luciferase renders it cytosolic (Gould *et al.*, 1989)), PTS2s apparently do not have a critical position relative to the N-terminus; the prepieces of rat thiolases A and B differ in that thiolase A has a 10-amino acid residue extension N-terminal to the PTS2 consensus sequence (Hijikata *et al.*, 1987; 1990; Bodnar *et al.*, 1990). PTS2 signals are recognized by the Pex7 family of peroxins (discussed in Section 1.6).

Some peroxisomal matrix proteins do not have PTS1- or PTS2-like sequences, which suggests that there may be more targeting signals to be found. Some poorly defined internal regions of proteins have also been implicated in peroxisomal targeting. Two large regions of *C. tropicalis* acyl-CoA oxidase were found to be important for peroxisomal targeting using

an *in vitro* import assay (Small *et al.*, 1988). Two proteins with apparent PTS1s also have internal targeting information. Deletion of the C-terminal tripeptides SKF and AKL from *S. cerevisiae* catalase A and carnitine acetyltransferase, respectively, does not alter their peroxisomal localization (Kragler *et al.*, 1993; Elgersma *et al.*, 1995). However, it is interesting to note that targeting of carnitine acetyltransferase via the internal PTS is dependent on a functional PTS1 receptor (Elgersma *et al.*, 1995). This has led to the speculation that accessory sequences on cargo proteins may exist to stabilize the receptor-cargo interaction, especially for cargo proteins containing a PTS that binds with low affinity to the receptor, and that these putative accessory sequences may be sufficient in some circumstances to effect an interaction with the receptor (Elgersma *et al.*, 1996b; Hettema *et al.*, 1999).

1.4.2 Translocation of proteins across the peroxisomal membrane

The conditions required for import of proteins into peroxisomes have been examined using mammalian cells that have been microinjected (Walton *et al.*, 1992; Soto *et al.*, 1993; Walton *et al.*, 1994), or permeabilized with streptolysin-O (Rapp *et al.*, 1993; Wendland and Subramani, 1993), as well as by import of *in vitro* translated proteins into purified peroxisomes (Imanaka *et al.*, 1987; Small and Lazarow, 1987) or glyoxysomes (Brickner *et al.*, 1997; Brickner and Olsen, 1998; Pool *et al.*, 1998). The combined findings of these studies suggest that the import of proteins into the matrix of peroxisomes (as judged by immunofluorescence following differential permeabilization of cellular membranes, or by protection from protease digestion) is a saturable process that appears to be independent of

a membrane potential and dependent on time, temperature, a PTS, ATP hydrolysis, Hsp70-type chaperones, and also involves peroxisome-associated factors sensitive to *N*-ethylmaleimide (NEM). Imanaka *et al.* (1987) reported that import of a radiolabelled protein into isolated rat liver peroxisomes was not enhanced by the addition of rat liver cytosol, but the source of their import substrate was a rabbit reticulocyte lysate, which was shown by Wendland and Subramani (1993) to be sufficient to supply cytosolic components essential for peroxisomal protein import in a permeabilized cell system. However, a purified, bacterially produced protein apparently does not require cytosolic factors for its import into isolated glyoxysomes (Pool *et al.*, 1998). Targeting (binding of the protein to the peroxisomal membrane) and import (translocation of the protein across the membrane) are separable processes, since the former can occur at low temperatures (0-4°C) and in the absence of ATP, while the latter requires temperatures of 26-37°C and ATP hydrolysis.

One remarkable feature of peroxisomal matrix protein import is that the translocation machinery is able to accommodate a wide variety of protein conformations. Oligomeric proteins (Glover *et al.*, 1994; McNew and Goodman, 1994; Elgersma *et al.*, 1996b; Lee *et al.*, 1997), stably folded proteins (Walton *et al.*, 1995; Häusler *et al.*, 1996), branched polypeptides (Walton *et al.*,1992) and even 9-nm gold particles decorated with PTS-1-containing peptides (Walton *et al.*, 1995) have all been successfully transported across the membranes of peroxisomes or other microbodies. This suggests that translocating proteins need not be in a completely unfolded conformation. Transport of oligomers is particularly interesting, because it opens up the possibility that some peroxisomal proteins may lack specific targeting information of their own and gain entry to the peroxisomal matrix by

"piggybacking" across the membrane with a protein that has a PTS. While this has not yet been formally demonstrated for an unaltered endogenous peroxisomal protein, it is possible that some regions of proteins that have been identified as "internal" PTSs may actually be protein-protein interaction domains required for this mode of import (Elgersma and Tabak, 1996). Import of oligomeric proteins has been demonstrated *in vivo* for both the PTS1- (McNew and Goodman, 1994;Elgersma *et al.*, 1996b; Lepier *et al.*, 1996; Lee *et al.*, 1997) and PTS2- (Glover *et al.*, 1994) dependent pathways. However, there is evidence to suggest that although import of oligomers is possible, it is not always essential (Lepier *et al.*, 1996) and may not be the most efficient method of translocation (Crookes and Olsen, 1998). Additionally, some proteins appear to be imported as monomers and subsequently multimerize in the peroxisomal matrix (Waterham *et al.*, 1997; Evers *et al.*, 1994; 1996).

The nature of the structure through which peroxisomal proteins must pass to reach the matrix is not known. It is also not known if all translocating proteins use the same translocon, or if there are multiple translocation machineries specific for different subsets of proteins. Hydrophilic channels composed of integral membrane proteins have been implicated in protein translocation across the membranes of other eukaryotic organelles (as well as the bacterial plasma membrane) (Schatz and Dobberstein, 1996), but none has yet been identified in peroxisomes. Although the translocation of oligomeric proteins might suggest a large pore like that found in the nuclear envelope (reviewed in Kessel *et al.*, 1988), the fact that *in vivo* the peroxisomal membrane is not freely permeable to small molecules (van Roermund *et al.*, 1995) suggests that the putative channel is either gated or forms only transiently. Additionally, large pores in peroxisomal membranes have never been reported from

morphological studies. Import of oligomeric proteins into peroxisomes has spawned the speculative theory that oligomerized proteins might be tolerated by the translocation apparatus because import occurs not via a proteinaceous channel through the membrane, but rather by the invagination and internalization of a region of the peroxisomal membrane. The membrane vesicle would then be triggered to open or dissolve in order to release its contents to the matrix (McNew and Goodman, 1994).

1.4.3 Targeting signals for integral membrane proteins

With one possible exception (Bodnar and Rachubinski, 1991), integral peroxisomal membrane proteins (PMPs) are also translated on free cytosolic polysomes (Fujiki *et al.*, 1984; Suzuki *et al.*, 1987; Bodnar and Rachubinski, 1991). PMP targeting is somewhat separable from, and seems to precede, matrix protein targeting, since most cells with defects in peroxisome biogenesis contain peroxisomal "ghosts"or other detectable structures that contain membrane proteins but lack some or most matrix proteins (for examples see Santos *et al.*, 1988a; Santos *et al.*, 1988b; Kalish *et al.*, 1996; Gould *et al.*, 1996; Erdmann and Blobel, 1996). The third PTS identified is a less well characterized sequence found on some PMPs (reviewed in Subramani, 1998; Hettema *et al.*, 1999). The mPTS, as it is known, is not similar in structure to either the PTS1 or the PTS2 signal. The mPTS was first defined for *C. boidinii* PMP47, a protein that is predicted to have six membrane-spanning domains (McCammon *et al.*, 1994). A 20 amino acid residue hydrophilic loop located between two transmembrane domains, and predicted to face the peroxisomal matrix, was both necessary and sufficient for peroxisomal targeting (Dyer *et al.*, 1996). Sequences within the first 45

amino acid residues of *H. polymorpha* (Baerends *et al.*, 1996), *P. pastoris* (Wiemer *et al.*, 1996), *S. cerevisiae* (Krause, 1995; cited in Soukupova *et al.*, 1999) and human (Kammerer *et al.*, 1998; Soukupova *et al.*, 1999) Pex3 proteins are sufficient to target reporter proteins efficiently to peroxisomes. The C-terminal tail of *S. cerevisiae* Pex15p also contains peroxisomal targeting information (Elgersma *et al.*, 1997). The regions of these proteins that can confer peroxisomal localization share a common motif with the consensus sequence (F/L)-(L/I/V)-X-(R/K)-X-(K/R)-X-(K/R)-X-(L/I)-(V/I/F/M)-X_{8-9}-(F/Y) (Elgersma *et al.*, 1997; Soukupova *et al.*, 1999). The targeting signals on *Pp*Pex3p[1], ScPex3p, HsPex3p and *Sc*Pex15p are, like the mPTS from *Cb*PMP47, also on regions of their respective proteins that are predicted to face the peroxisomal matrix. A receptor for the mPTS has not yet been identified. The first 25 amino acid residues of Pex22p from *P. pastoris* can target a reporter protein to peroxisomes (Koller *et al.*, 1999). This region is predicted to face the matrix and includes a stretch of positively charges residues, but it does not share extensive sequence similarity with the consensus mPTS and may therefore represent a distinct peroxisomal membrane targeting signal.

1.4.4 ER involvement in peroxisomal membrane protein trafficking

Evidence for ER involvement in PMP transport comes from the study of *Y. lipolytica* proteins Pex2p (an integral PMP (Eitzen *et al.*, 1995)) and Pex16p (a peripheral PMP

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1 By convention (Distel *et al.*, 1996), the two letters preceding the name of a protein or gene designate the genus (upper case) and species (lower case) of the organism in which it was identified.

associated with the inner face of the peroxisomal membrane (Eitzen *et al.*, 1997)). These proteins normally traffic through the ER on their way to peroxisomes and are present in peroxisomes with ER-specific modifications (Titorenko and Rachubinski, 1998b). However, *Yl*Pex2p and *Yl*Pex16p are delayed or blocked in their exit from the ER in some mutants of the secretory pathway or peroxisome biogenesis, respectively. No peroxisomal matrix proteins tested were detected in ER-enriched fractions, suggesting that this trafficking route may be specific for PMPs in *Y. lipolytica*.

Interestingly, the minimal regions of *Hp*Pex3p and *Sc*Pex15p that contain the mPTS seem to also contain information for targeting to the ER. An overproduced reporter protein with the first 16 amino acid residues of *Hp*Pex3p fused to its N-terminus is inefficiently targeted to peroxisomes, primarily accumulating in ER membranes surrounding the nucleus (Baerends *et al.*, 1996). Overproduction of *Sc*Pex15p or of a reporter protein fused to the C-terminal 82 amino acid residues of *Sc*Pex15p resulted in the localization of the proteins to profoundly proliferated ER membranes that also surround the nucleus (Elgersma *et al.*, 1997). However, there is recent evidence that, at least in *S. cerevisiae*, this effect may be non-specific, and that the ER may be a sink for overproduced hydrophobic proteins (Stroobants *et al.*, 1999; Hettema *et al.*, 1999).

1.4.5 Integration of proteins into the peroxisomal membrane

Insertion of PMPs has been studied using *in vitro* import into isolated peroxisomes (Diestelkötter and Just, 1993; Imanaka *et al.*, 1996; Pause *et al.*, 1997) or semi-intact cells (Diestelkötter and Just, 1993). The results of these studies suggest that, like matrix protein

import, PMP insertion (as judged by resistance to carbonate extraction or digestion by a specific protease) is also time- and temperature-dependent, and involves cytosolic factors (one of which is probably the TCP1 ring complex chaperonin (Pause *et al.*, 1997)) as well as proteins at the peroxisome surface. However, in contrast to matrix protein import, PMP insertion is not dependent on ATP hydrolysis and is not inhibited by treatment with NEM. Binding to the membrane surface and insertion have been found to be separable events for rat PMP22, since binding occurs at 0°C, while insertion occurs at 26°C (Diestelkötter and Just, 1993). However, binding of rat PMP70 to isolated peroxisomes at 0°C was not detected (both binding and insertion were observed to occur at 26°C) (Imanaka *et al.*, 1996), suggesting that slightly different mechanisms may be at work for the integration of these two proteins.

It is not currently known if PMPs use the same putative translocon(s) as matrix proteins. Also, it is difficult to see how the theory of protein import by membrane invagination (McNew and Goodman, 1994) could be applied to PMPs.

1.5 Model systems for the study of peroxisome biogenesis at the molecular level

As discussed in the preceding sections, initial investigations of peroxisome biogenesis focussed on the requirements for targeting of peroxisomal proteins and their import into the matrix or integration into the membrane. While these investigations began to establish the basic conditions that promote peroxisomal protein import/integration, they did little to elucidate the molecular mechanisms involved in this process and could not address the more preliminary events in peroxisome formation.

By far the most informative approach in the search for peroxins (proteins directly involved in peroxisome biogenesis and encoded by *PEX* genes (Distel *et al.*, 1996)) has been the creation and characterization of mutants of peroxisome biogenesis (*pex* mutants) in genetically tractable systems. Several species of yeast as well as Chinese hamster ovary (CHO) cells have been employed for this purpose. This strategy is possible, because under certain conditions peroxisomes are dispensable for cell survival, thus allowing the maintenance of mutant cell lines and obviating the need for temperature-sensitive mutations that would be necessary if the organelle were essential under all conditions. The first *pex* mutants in yeast were identified in *S. cerevisiae* (Erdmann *et al.*, 1989). This screen exploited the fact that peroxisomes are the exclusive site of β-oxidation in yeast (Kunau *et al.*, 1988), so colonies unable to grow on media with oleic acid as the sole carbon source are likely to have a defect in peroxisome function. Peroxisome assembly mutants were distinguished from β-oxidation or metabolite transport mutants by screening the mutants for abnormal peroxisomal morphology in electron micrographs, as well as by the mislocalization of peroxisomal marker enzymes after subcellular fractionation. The defective genes were isolated by transforming the mutants with a genomic DNA library and assaying for functional complementation of the oleate growth defect (Erdmann *et al.*, 1991; Höhfeld *et al.*, 1991; Wiebel and Kunau, 1992). Similar negative selection strategies have been used to identify *pex* mutants in the yeasts *H. polymorpha* (Cregg *et al.*, 1990; Nuttley *et al.*, 1995), *P. pastoris* (Gould *et al.*, 1992; Liu *et al.*, 1992) and *Y. lipolytica* (Nuttley *et al.*, 1993), using a growth assay on methanol or oleic acid medium, as appropriate, as the primary screen. Other screens of mutated cells employing different selection strategies (reviewed in Elgersma and Tabak,

1996; Subramani 1998) have been used to identify *pex* mutants in yeasts and CHO cells. Peroxins have also been found by reverse genetics (*e.g.* Erdmann and Blobel, 1995), as well as by two-hybrid interactions (*e.g.* Brocard *et al.*, 1994). These two techniques allow the identification of *PEX* genes that may be essential, and which would therefore not be isolated using a growth screen. Other *PEX* genes have been identified based on their sequence similarities to genes cloned from other species using nucleic acid hybridization (Shimozawa *et al.*, 1992), degenerate PCR (*e.g.* Heyman *et al.*, 1994) or database searching (*e.g.* Dodt *et al.*, 1995). The subsequent identification of human *PEX* gene orthologues, several of which have been found to be mutated in patients with peroxisome biogenesis disorders (reviewed in Wanders, 1999), demonstrate the validity of using yeast and CHO *pex* mutants as models for these diseases. Table 1-1 lists *PEX* genes identified to date and key features of the peroxins they encode.

At least 23 different proteins have been identified as having a role in peroxisome assembly. These include proteins hypothesized to function in PTS recognition (Pex5p, Pex7p), docking and binding of PTS receptor-cargo protein complexes at the membrane (Pex10p, Pex12p, Pex13p, Pex14p, Pex17p), organelle proliferation (Pex11p, Pex16p), insertion of membrane proteins (Pex3p, Pex19p), molecular chaperoning (Pex20p), recruitment of membrane lipids (Pex2p) and membrane fusion events (Pex1p and Pex6p). However, there is only direct evidence for function for a few of these. For many *PEX* genes, encoding widely divergent proteins, the effect of their mutation is abnormal peroxisome morphology and mislocalization of a subset of peroxisomal proteins to the cytosol. This phenotype is not particularly informative for assigning functions to the gene products, since

mislocalization of matrix proteins does not necessarily imply that the affected peroxin participates in protein translocation; for example, the import defect could be a downstream effect of the failure to correctly proliferate the organelle or assemble membrane components. The best functionally characterized peroxins are Pex5p and Pex7p, which are discussed in Section 1.6.

Table 1-1. Proteins involved in peroxisome biogenesis

Abbreviations: AAA, ATPases associated with diverse cellular activities; ARF, ADP-ribosylation factor; CG, complementation group of human peroxisome biogenesis disorders; PMP, peroxisomal membrane protein; SH3, Src homology 3; TPR, tetratricopeptide repeat.

Peroxin[a]	Features	References
Pex1p	AAA family ATPase; vesicle-associated and/or cytosolic; interacts with Pex6p; required for ER-to-peroxisome transit of Pex2p and Pex16p in *Y. lipolytica*; human *PEX1* complements CG1[b]	Erdmann *et al.*, 1991; Heyman *et al.*, 1994; Portsteffen *et al.*, 1997; Reuber *et al.*, 1997; Faber *et al.*, 1998; Geisbrecht *et al.*, 1998; Tamura *et al.*, 1998a, 1998b; Titorenko and Rachubinski, 1998b
Pex2p	C_3HC_4 zinc-binding integral PMP; glycosylated in *Y. lipolytica*; human *PEX2* complements CG10[b]	Tsukamoto *et al.*, 1991; Shimozawa *et al.*, 1992; Berteaux-Lecellier *et al.*, 1995; Eitzen *et al.*, 1996; Titorenko *et al.*, 1996; Waterham *et al.*, 1996; Faust and Hatten, 1997; Titorenko and Rachubinski, 1998b
Pex3p	integral PMP; interacts with Pex19p	Höhfeld *et al.*, 1991; Baerends *et al.*, 1996, Wiemer *et al.*, 1996; Götte *et al.*, 1998; Kammerer *et al.*, 1998; Snyder *et al.*, 1999; Soukupova *et al.*, 1999
Pex4p	ubiquitin-conjugating enzyme; peripheral PMP associated with outer face of peroxisomal membrane or cytosolic and vesicle-associated; interacts with Pex22p; may be important for Pex5p recycling in *H. polymorpha*	Wiebel and Kunau, 1992; Crane *et al.*, 1994; van der Klei *et al.*, 1998; Koller *et al.*, 1999
Pex5p	PTS1 import receptor; 7 C-terminal TPR motifs; cytosolic and/or peroxisome-associated; two genes identified in tobacco; short and long isoforms in mammals generated from alternative splicing of mRNA; cycling receptor in humans; human *PEX5* complements CG2[b]	McCollum *et al.*, 1993; van der Leij *et al.*, 1993; Dodt *et al.*, 1995; Fransen *et al.*, 1995; Nuttley *et al.*, 1995; van der Klei *et al.*, 1995; Wiemer *et al.*, 1995; Baes *et al.*, 1997; Brickner *et al.*, 1998; Braverman *et al.*, 1998; Kragler *et al.*, 1998; Otera *et al.*, 1998; Wimmer *et al.*, 1998; de Walque *et al.*, 1999

Pex6p	AAA family ATPase; peripheral PMP, cytosolic or vesicle-associated; interacts with Pex1p; required for ER-to-peroxisome transit of Pex2p and Pex16p in *Y. lipolytica*; human *PEX6* complements CG4[b]	Spong and Subramani, 1993; Voorn-Brouwer *et al.*, 1993; Nuttley *et al.*, 1994; Tsukamoto *et al.*, 1995; Yahraus *et al.*, 1996; Faber *et al.*, 1998; Geisbrecht *et al.*, 1998; Tamura *et al.*, 1998b; Titorenko and Rachubinski, 1998b
Pex7p	cytosolic and/or peroxisomal PTS2 import receptor; 6 WD-40 repeats; human *PEX7* complements CG11[b] (RCDP-1)	Marzioch *et al.*, 1994; Zhang and Lazarow, 1995, 1996; Rehling *et al.*, 1996; Braverman *et al.*, 1997; Motley *et al.*, 1997; Purdue *et al.*, 1997; Elgersma *et al.*, 1998; Schumann *et al.*, 1999.
Pex8p	peroxisomal protein; localized to matrix or associated peripherally with inner side of peroxisomal membrane	Waterham *et al.*, 1994; Liu *et al.*, 1995; Smith *et al.*, 1997
Pex9p	integral PMP	Eitzen *et al.*, 1995
Pex10p	C_3HC_4 zinc-binding integral PMP; interacts with Pex19p; required for human Pex5p recycling; human Pex10p interacts with Pex12p; human *PEX10* complements CG7[b]	Kalish *et al.*, 1995; Tan *et al.*, 1995; Dodt and Gould, 1996; Okumoto *et al.*, 1998; Warren *et al.*, 1998; Snyder *et al.*, 1999; Chang *et al.*, 1999
Pex11p	integral or peripheral PMP; promotes proliferation; no apparent involvement in protein import; α (inducible) and β (constitutive) isoforms identified in mammals; may recruit ARF and coatomer	Erdmann and Blobel, 1995; Marshall *et al.*, 1995; Sakai *et al.*, 1995; Abe and Fujiki, 1998; Abe *et al.*, 1998; Lorenz *et al.*, 1998; Passreiter *et al.*, 1998; Schrader *et al.*, 1998
Pex12	C_3HC_4 zinc-binding integral PMP; interacts with Pex5p and Pex10p; required for human Pex5p recycling; human *PEX12* complements CG3[b]	Dodt and Gould, 1996; Kalish *et al.*, 1996; Chang *et al.*, 1997; Okumoto and Fujiki, 1997; Okumoto *et al.*, 1998; Chang *et al.*, 1999
Pex13p	SH3 domain-containing integral PMP; part of import receptor docking complex; interacts with Pex5p, Pex7p, Pex14p; human *PEX13* complements CGH[c]	Elgersma *et al.*, 1996; Erdmann *et al.*, 1996; Gould *et al.*, 1996; Björkman *et al.*, 1998; Fransen *et al.*, 1998; Girzalsky *et al.*, 1999; Liu *et al.*, 1999; Shimozawa et al., 1999
Pex14p	integral or peripheral PMP; phosphorylated in *H. polymorpha*; part of import receptor docking complex; interacts with Pex5p, Pex7p, Pex13p, Pex17p	Albertini *et al.*, 1997; Brocard *et al.*, 1997; Komori *et al.*, 1997; Fransen *et al.*, 1998; Komori *et al.*, 1999; Schliebs *et al.*, 1999; Shimizu *et al.*, 1999; Will *et al.*, 1999
Pex15p	integral PMP, phosphorylated	Elgersma *et al.*, 1997
Pex16p	integral or peripheral PMP; involved in proliferation; glycosylated in *Y. lipolytica*; human *PEX16* complements CG9[b]	Eitzen *et al.*, 1997; Honsho *et al.*, 1998; Lin *et al.*, 1999; South *et al.*, 1999
Pex17p	peripheral PMP associated with outside of peroxisomal membrane; part of import receptor docking complex; interacts with Pex14p	Huhse *et al.*, 1998
Pex18p	PTS2-specific import factor; primarily cytosolic; interacts with Pex7p; related to Pex21p	Purdue *et al.*, 1998

Pex19p	farnesylated protein (except *P. pastoris* orthologue); cytosolic and peroxisome-associated; interacts with Pex3p and Pex10p; human *PEX19* complements CGJ[c]	James *et al.*, 1994; Götte *et al.*, 1998; Matsuzono *et al.*, 1999; Snyder *et al.*, 1999
Pex20p	thiolase-specific (PTS2-specific?) import factor; cytosolic and peripherally associated with outside of membrane of high-speed pelletable peroxisomes	Titorenko *et al.*, 1998
Pex21p	PTS2-specific import factor; cytosolic; interacts with Pex7p; related to Pex18p	Purdue *et al.*, 1998
Pex22p	integral PMP; anchors Pex4p to the peroxisomal membrane	Koller *et al.*, 1999
Pex23p	integral PMP	Brown *et al.*, 2000

[a] Peroxin nomenclature according to Distel *et al.*, 1996.
[b] Nomenclature of Kennedy Krieger Insitute (USA) research group (Wanders, 1999).
[c] Nomenclature of Gifu University School of Medicine (Japan) research group (Wanders, 1999).

1.6 PTS1 and PTS2 receptors

Pex5p and Pex7p have been shown to be receptors for PTS1 and PTS2, respectively. The Pex5 family is characterized by a tetratricopeptide repeat (TPR) domain in the C-terminal half of the protein that is involved in PTS1 binding (Brocard *et al.*, 1994; Dodt *et al.*, 1995; Terlecky *et al.*, 1995; Wiemer *et al.*, 1995; Kragler *et al.*, 1998). TPR domains consist of tandem arrays of a degenerate 34-amino acid residue motif and have been implicated in protein-protein interactions for proteins with many diverse cellular roles (Goebl and Yanagida, 1991). The hallmark of the Pex7 peroxin is the WD-40 motif, which has also been implicated in protein-protein interactions, including those with TPR-containing proteins (Goebl and Yanagida, 1991; van der Voorn and Ploegh, 1992).

As Table 1-1 indicates, the subcellular localization of Pex5p and Pex7p is somewhat controversial, and has led to several hypotheses concerning their mechanisms of function

(reviewed in Erdmann *et al.*, 1997; Waterham and Cregg, 1997). Tight association with the cytoplasmic face of the peroxisomal membrane implies a static receptor that binds polypeptides at the membrane surface prior to import. A combined cytosolic and peroxisome-associated localization suggests that Pex5p and Pex7p may be more dynamic, binding their recognition sequences in the cytosol, docking at the peroxisomal membrane and releasing their cargo protein for subsequent import by other factors. Partial localization inside peroxisomes led to the extended shuttle hypothesis, whereby the receptor accompanies cargo proteins through the translocon, disengages from the cargo in the matrix and returns to the cytosol. Finally, localization of a receptor exclusively in the peroxisomal matrix of peroxisomes has led to the suggestion that PTS receptors can operate from inside peroxisomes to actively pull proteins into the organelle. A model incorporating all these disparate findings has been proposed, which hypothesises that PTS receptors cycle between the cytosol and peroxisomes, and that changing cellular conditions may influence the relative distributions of the receptors (Rachubinski and Subramani, 1995). Additionally, although the proteins encoded by the *PEX5* and *PEX7* genes have high amino acid residue sequence similarities within their respective families, it is not inconceivable that their functions may differ as individual organisms may have evolved slightly different mechanisms for maximizing efficient matrix protein import.

Although Pex5p- and Pex7p-dependent protein targeting are independent processes in lower eukaryotes, both PTS receptors interact (directly or indirectly) with a common network of peroxins at the peroxisomal membrane that includes Pex13p (Elgersma *et al.*, 1996a; Erdmann and Blobel, 1996; Gould *et al.*, 1996; Girzalsky *et al.*, 1999), Pex14p

(Albertini *et al.*, 1997; Brocard *et al.*, 1997; Komori *et al.*, 1997; Girzalsky *et al.*, 1999) and Pex17p (Huhse *et al.*, 1998). These three peroxins are hypothesized to be part of a PTS receptor docking complex.

The loss of function of either PTS receptor leads to disease states in humans, causing NALD and ZS in the case of Pex5p mutation (Dodt *et al.*, 1995) and RCDP-1 in the case of Pex7p deficiency (Braverman *et al.*, 1997; Motley *et al.*, 1997; Purdue *et al.*, 1997). Interestingly, import of proteins by the PTS1 and PTS2 pathways is not entirely independent in mammalian cells. The human (Dodt *et al.*, 1995; Fransen *et al.*, 1995; Wiemer *et al.*, 1995; Braverman *et al.*, 1998), mouse (Braverman *et al.*, 1998) and Chinese hamster (Otera *et al.*, 1998) *PEX5* genes each encode two forms of the protein that differ by the presence or absence of a 37-amino acid residue insertion N-terminal to the TPR domain, apparently generated by alternative splicing of the *PEX5* transcript. The short form of Pex5p can functionally complement the PTS1 import defect in *pex5* mutant cell lines, but only the long form of the protein can also rescue the PTS2 import defect that is an additional feature of some *pex5* cell lines (Braverman *et al.*, 1998; Otera *et al.*, 1998). Mammalian orthologues of Pex13p (Gould *et al.*, 1996; Fransen *et al.*, 1998; Liu *et al.*, 1999; Shimozawa *et al.*, 1999) and Pex14p (Fransen *et al.*, 1998; Will *et al.*, 1999; Shimizu *et al.*, 1999; Schliebs *et al.*, 1999) have also been identified. Recently, human Pex10p and Pex12p have been shown to interact, and to be involved in an event (or events) downstream of the initial docking step (Chang *et al.*, 1999). The molecular processes that occur at the peroxisomal membrane are not yet known.

1.7 Scope and approach of this thesis

At the time this project was initiated, the genetic analysis of peroxisome biogenesis was in its infancy. A total of only five genes from *S. cerevisiae* (Erdmann *et al.*, 1991; Höhfeld *et al.*, 1991; Wiebel and Kunau, 1992), rat (Tsukamoto *et al.*, 1991) and human (Shimozawa *et al.*, 1992), representing four peroxin families, had been reported. The Rachubinski laboratory had developed an effective screening methodology for mutants of peroxisome biogenesis in the yeast *Y. lipolytica* (Nuttley *et al.*, 1993). This genetically amenable species offers an advantage over the traditionally studied yeast *S. cerevisiae*, in that *Y. lipolytica* has a greater peroxisome proliferative response when grown in oleic acid medium, thus easily allowing the isolation of large amounts of peroxisomal material. The original screen had identified only two *pex* mutants in this yeast; many more would be needed to more completely understand the molecular mechanisms of peroxisome assembly. The work presented herein describes the creation of a series of mutants, the biochemical characterization of one of these, the identification of the complementing gene and an analysis of the function of its product.

CHAPTER 2

Materials and Methods

2.1 Materials

All reagents were of the highest quality available and, where required, were used according to the manufacturers' specifications, unless otherwise indicated.

2.1.1 Chemicals and reagents

acetyl-CoA, sodium salt	Sigma
acrylamide	Gibco/BRL
acrylamide solution, ExplorER	J.T. Baker
acrylamide solution, Long Ranger	J.T. Baker
agar	Difco
agarose, electrophoresis grade	Gibco/BRL
agarose, NuSieve GTG	FMC BioProducts
agarose, SeaKem GTG	FMC BioProducts
albumin, bovine serum (BSA)	Roche
L-amino acids	Sigma
ammonium persulphate	BDH
ampicillin	Sigma
antipain	Roche
aprotinin	Roche
benzamidine hydrochloride	Sigma
Bio-Rad protein assay dye reagent	Bio-Rad
B-PER bacterial protein extraction reagent	Pierce
Brij-35 (polyoxyethylene 23-lauryl ether)	Sigma
bromophenol blue	BDH
CSM-leu, ura (complete supplements mixture minus leucine and uracil)	BIO 101
CSM-ura (complete supplements mixture minus uracil)	BIO 101
Coomassie Brilliant Blue R-250	ICN
cytochrome *c*, horse heart	Sigma
DEPC (diethyl pyrocarbonate)	Sigma
DNA, from salmon testes, sodium salt	Sigma
DTT (dithiothreitol)	ICN
EDTA (ethylenediaminetetraacetic acid)	Sigma
Ficoll	Amersham-Pharmacia
5-FOA (5-fluoroorotic acid)	Toronto Research Chemicals
Freund's adjuvant (complete and incomplete)	Sigma
formamide	BDH

formaldehyde	BDH
glutathione, reduced	Sigma
glutathione-Sepharose	Amersham-Pharmacia
glycerol	BDH
glyoxylic acid, sodium salt	Sigma
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)	Roche
hydrogen peroxide solution, 30% (w/v)	Sigma
β-hydroxybutyryl-CoA	Sigma
IPTG (isopropyl β-D-thiogalactopyranoside)	Vector Biosystems
isocitric acid, trisodium salt	Sigma
leupeptin	Roche
NAD^+ (β-nicotinamide adenine dinucleotide, free acid)	Roche
N,N'-methylenebisacrylamide	Gibco/BRL
NP-40 (Nonidet P-40)	BDH
NTG (1-methyl-3-nitro-1-nitrosoguanidine)	Sigma
2-mercaptoethanol	BDH
MES (2-[N-morpholino]ethanesulphonic acid)	Sigma
MOPS (3-[N-morpholono]propanesulphonic acid)	Sigma
oleic acid	Fisher
PEG-8000 (polyethylene glycol, average molecular weight 8,000)	Sigma
pepstatin A	Sigma
peptone	Difco
phenol, buffer-saturated	Gibco/BRL
phenylhydrazine hydrochloride	Sigma
PMSF (phenylmethylsulphonyl fluoride)	Roche
polyvinylpyrrolidone	Sigma
Ponceau S	Sigma
potassium glutamate	Fluka
PPO (2,5-diphenyloxazole)	Sigma
protein A-Sepharose	Amersham-Pharmacia
RNasin	Promega
Sephadex G-50	Amersham-Pharmacia
sodium dithionite (sodium hydrosulphite)	Sigma
SDS (sodium dodecyl sulphate)	Sigma
sodium fluoride	Sigma
sodium sulphite	Sigma
sorbitol	BDH
sucrose	BDH
TCA (trichloroacetic acid)	BDH
TEMED (*N,N,N',N'*-tetramethylethylene-diamine)	Gibco/BRL

Tris (tris(hydroxymethyl)aminomethane)	Roche
Triton X-100	Sigma
tryptone	Difco
Tween 20 (polyoxyethylenesorbitan monolaurate)	Sigma
Tween 40 (polyoxyethylenesorbitan monopalmitate)	Sigma
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)	Vector Biosystems
xylene cyanol FF	Sigma
yeast extract	Difco
YNB (yeast nitrogen base without amino acids)	Difco

2.1.2 Enzymes

2.1.2.1 DNA modifying enzymes

CIP (calf intestinal alkaline phosphatase)	NEB
DNA ligase, T4	Gibco/BRL, NEB, Roche
DNA polymerase I, *Escherichia coli*, Klenow fragment	NEB
DNA polymerase, T4	NEB
DNase I, bovine pancreas	Sigma
restriction endonucleases	Gibco/BRL, NEB, Promega, Roche
polynucleotide kinase, T4	NEB
Taq polymerase (*Thermus aquaticus* DNA polymerase)	Roche

2.1.2.2 Other enzymes

RNase A (ribonuclease A), bovine pancreas	Sigma, Roche
trypsin	Roche
Zymolyase 100T	ICN

2.1.3 Multicomponent systems

QIAprep MiniPrep Kit	Qiagen
Qiagen Plasmid Midi Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
Random Primers Labelling Kit	Roche
Ready-to-Go PCR Beads	Amersham-Pharmacia

Sculptor DNA Mutagenesis Kit	Amersham-Pharmacia
Sequenase Version 1.0 DNA Sequencing Kit	USB
Sequenase Version 2.0 DNA Sequencing Kit	USB
TNT T7 Coupled Reticulocyte Lysate System	Promega

2.1.4 Radiochemicals and detection systems

α-[^{32}P]dATP (3,000 Ci/mmol, 10 μCi/μL, Redivue)	Amersham-Pharmacia
L-[^{35}S]methionine (1,175 Ci/mmol, 10 mCi/mL)	ICN
enhanced chemiluminescence detection kits for immunoblotting and nucleic acid blotting, horseradish peroxidase-linked	Amersham-Pharmacia
Immobilon P	Millipore
nitrocellulose	Amersham-Pharmacia, Bio-Rad
X-ray film	Kodak

2.1.5 Molecular size standards

1 kb DNA ladder (75-12,216 bp)	Gibco/BRL
25 bp DNA ladder (25-500 bp)	Gibco/BRL
prestained markers for SDS-PAGE (6.5, 16.5, 25, 32.5, 47.5, 62, 83, 175 kD)	NEB

2.1.6 Plasmid vectors

2.1.6.1 *E. coli* vectors

pBluescriptSKII(-)	Stratagene
pGEM5Zf(+)	Promega
pGEM7Zf(+)	Promega
pGEX-4T1	Amersham-Pharmacia
pMAL-c2	NEB
pRcCMV	Invitrogen

2.1.6.2 *E. coli*/yeast shuttle vectors

pINA443 (*Y. lipolytica*)	Dr. Claude Gaillardin, Institut National Agronomique Paris-Grignon, Thiverval-Grignon, France (*ARS68, URA3*)
pINA445 (*Y. lipolytica*)	Dr. Claude Gaillardin (Nuttley *et al.*, 1993)

pSG464 (*P. pastoris*)	Dr. Suresh Subramani, University of California, San Diego, La Jolla, California (Gould *et al.*, 1992)

2.1.7 Antibodies

2.1.7.1 Commercially obtained antibodies

anti-*S. cerevisiae* glucose-6-phosphate dehydrogenase (from rabbit)	Sigma
anti-guinea pig IgG (from goat), horseradish peroxidase-conjugated	Sigma
anti-rabbit IgG (from donkey), horseradish peroxidase-conjugated	Amersham-Pharmacia
anti-guinea pig IgG (from donkey), rhodamine (TRITC)-conjugated	Jackson
anti-rabbit IgG (from donkey), fluorescein (FITC)-conjugated	Jackson
anti-rabbit IgG (from donkey), rhodamine (TRITC)-conjugated	Jackson

2.1.7.2 Other antibodies

The production of anti-*Yl*Pex5p antibodies is described in Section 2.12. Rabbit anti-SKL serum was prepared by Dr. John Glover (McMaster University, Hamilton, Ontario) against the peptide NH_2-CRYHLKPLQSKL-COOH conjugated to keyhole limpet hemocyanin, as described (Gould *et al.*, 1990b). Guinea pig antiserum to *Y. lipolytica* thiolase was prepared by Dr. Gary Eitzen and Ms. Eileen Reklow against a fusion protein consisting of *E. coli* maltose binding protein (MBP) and *Y. lipolytica* thiolase (Eitzen *et al.*, 1996). Rabbit antibodies to *S. cerevisiae* peroxisomal acyl-CoA oxidase that also recognize *Y. lipolytica* acyl-CoA oxidase 1 were a gift from Dr. Joel Goodman (University of Texas, Dallas, Texas). Rabbit antibodies to *P. pastoris* Pex5p (McCollum *et al.*, 1993) were a gift from Dr. Suresh Subramani. Rabbit antibodies to *Y. lipolytica* Kar2p (Titorenko *et al.*, 1997)

were a gift from Dr. David Ogrydziak (University of California, Davis, California).

2.1.8 Oligodeoxyribonucleotides

Table 2-1 details the sequences and applications of oligodeoxyribonucleotides (oligonucleotides) used in this study. Oligonucleotides were synthesised at the DNA Sequencing Facility, Department of Biochemistry, University of Alberta or using a Beckman Oligo 1000M synthesizer at the Department of Cell Biology, University of Alberta.

Table 2-1 Synthetic oligodeoxyribonucleotides

Name	Sequence[a,b]	Application
PEX5#1	AGAACTGGAACGCCGAG	*PEX5* sequencing primer
PEX5#2	CTGCCGTTGGCAAGGGT	"
PEX5#3	TGCCGACTACCAGTTCG	"
PEX5#4	AGCCATGTTGGCACTCA	"
PEX5#5	TCCGTCAGGGGACAGTT	"
PEX5#6	CAGGTTAGTGGACTGGT	"
PEX5#7	ACTAATCTGTGACTCTT	"
PEX5#9	CCAGCGCGGTAGTTGCA	"
PEX5#10	GCCTCGGCTCAAATTCC	"
PEX5#11	CCGCTGAGGACCGATCC	"
PEX5#12	ACCAACGAAGGATACGAG	"
PEX5#13	CTCTTCTGTGGAATAGAC	"
PEX5#14	AAGGTTGGAAACGGAATG	"
PEX5#15	TAAACTGTGACTCTTGCC	"
PEX5#16	GGATCGGTCCTCAGCGG	"
PEX5#17	TGGAACGATGCATCAAGC	"
PEX5#18	CAAGGTTGGAAACGGAATGG	"

PEX5#19	ATTAAATACCACTCCCTGAC	"
PEX5#20	TCACCAAACACACCGCTG	"
PEX5#21	CTAACGCACGAGTTGTCG	"
PEX5#22	AAGGAGGACCCTGCTATC	"
PEX5#23	GTGCGTGCTCGATACAAC	"
PEX5#24	CTTGCTTTGGTGGGTCTC	"
PEX5#25	TGTCGCATCTGCTCGTAG	"
32RI5'	ATTGAATTCCAGCGCGGTAGTTGC	5' PCR primer to amplify entire *PEX5* gene
32RI3'	ATTGAATTCTAATAACCTTGGGCCTCC	3' PCR primer to amplify entire *PEX5* gene
G455D	CGTTTCCGTAGAACAGAACATCTAGACCAACT TGGACATCAGCG	*In vitro* mutagenesis to generate *PEX5* mutant G455D
KK	CCTCTCATAAACGACATGGTGGTACCTCCTGC TTGCTTTGG	*In vitro* mutagenesis to create consensus mammalian translation initiation site for *PEX5* open reading frame
KKBT	ATTGGTACCACTATGGGTATGGGGGGAATGC CCATG	5' PCR primer to create *PEX5* mutant B
KKCT	ATTGGTACCACCATGGAGCACCCTGATCCCTTC	5' PCR primer to create *PEX5* mutant C
KKET	ATTGGTACCACCATGGGCAACGAGGATAAGT TCCAG	5' PCR primer to create *PEX5* mutant E
KKREV	GGCCAATAATCCATGGCTGC	3' PCR primer to create *PEX5* mutants B, C and E
BIAT	ATTTACGTACTAGTGCTCCATGAACTGGTTG	3' PCR primer to create *PEX5* mutant A
BIDT	ATTTACGTACTATCGGGCCTGGTCCACAACC	3' PCR primer to create *PEX5* mutant D
BIFT	ATTTACGTACTACTCAACCTTGTGCATGGAC	3' PCR primer to create *PEX5* mutant F
EQ1	GATCCCCGC	Annealing/ligation to create pGEXQ
EQ2	AATTGCGGG	"
SKL1	TCGATCCAAGCTTTGA	Annealing/ligation to create pGEXQ-SKL
SKL2	GGCCTCAAAGCTTGGA	"

LKS1	TCGACTTAAGTCCTGA	Annealing/ligation to create pGEXQ-LKS
LKS2	GGCCTCAGGACTTAAG	"
AKI1	TCGAGCAAAGATCTGA	Annealing/ligation to create pGEXQ-AKI
AKI2	GGCCTCAGATCTTTGC	"
SKLS1	TCGATCCAAGCTTTCCTGA	Annealing/ligation to create pGEXQ-SKLS
SKLS2	GGCCTCAGGAAAGCTTGGA	"
M13FP	GTAAAACGACGGCCAGT	M13 forward sequencing primer for analysis of pGEM- or pBluescript-based plasmids
M13RP	CAGGAAACAGCTATGAC	M13 reverse sequencing primer for analysis of pGEM- or pBluescript-based plasmids
pRcCMV1	TATTGTCTCATGAGCGG	Primer for sequencing *BglII* site of pRcCMV
pRcCMV2	GTTCAATCATGCGAAACG	Primer for sequencing *StuI* site of pRcCMV
4T1-871	GCTGGCAAGCCAGCTTTGG	Primer for sequencing fusion site of pGEXQ-based plasmids

[a] All sequences are written 5' to 3'.
[b] Relevant restriction endonuclease sites or compatible overhangs are underlined (see appropriate sections of this chapter for details).

2.1.9 Commonly used buffered solutions

Table 2-2 details the compositions of some commonly used buffered solutions. Unless otherwise indicated, all solutions of solids that are expressed in percentages refer to weight per volume.

2.2 Microorganisms and culture conditions

2.2.1 Bacterial and bacteriophage strains and culture conditions

The *Escherichia coli* strain DH5α (F^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deo*R

Table 2-2 Buffered solutions

Solution	Composition	Reference
50 x Denhardt's	1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA	Maniatis *et al.*, 1982
5 x KGB	0.5 M potassium glutamate, 125 mM Tris-acetate, pH 7.6, 50 mM magnesium acetate, 250 μg BSA/mL, 2.5 mM 2-mercaptoethanol	Hanish and McClelland, 1988
P8BB	20 mM HEPES-KOH, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EDTA, 2 mM DTT	McCollum *et al.*, 1993
PBS	137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mMKH_2PO_4, pH 7.3	Pringle *et al.*, 1991
20 x SSC	3 M NaCl, 0.3 M trisodium citrate, pH 7.0	Maniatis *et al.*, 1982
10 x TBE	0.89 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA	Maniatis *et al.*, 1982
TBST	20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (w/v) Tween 20	Huynh *et al.*, 1985
TE	10 mM Tris-HCl, pH 7.0-8.0 (as appropriate), 1 mM EDTA	Maniatis *et al.*, 1982

*rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1) (Gibco/BRL) was routinely used for the amplification of plasmids (Sections 2.3.1 and 2.4.1). *E. coli* TG1 (K12 Δ(*lac-pro*) *sup*E *thi hsd*D5/F'*tra*D36 *pro*A$^+$B$^+$ *lac*Iq *lac*ZΔM15) (Amersham-Pharmacia) cells were infected with bacteriophage M13K07 (Promega) to generate single-stranded DNA (Sections 2.7.1 and 2.7.2). *E. coli* BLR-DE3 (F$^-$ *omp*T *hsd*S_B(r_B^-, m_B^+) *gal dcm lon* (*srl-rec*A)*306::Tn10* (DE3)) (Novagen) was used for the production of maltose binding protein (MBP) and glutathione-S-transferase (GST) chimeric proteins (Sections 2.12.1, 2.15.2 and 2.15.3). Bacteria were grown in a rotary shaker and, unless otherwise indicated, growth was at 37°C. Table 2-3 lists the bacterial culture media employed in this study.

Table 2-3 Bacterial culture media

Medium	Composition	Reference
2 x YT[a]	1.6 % tryptone, 1% yeast extract, 0.5% NaCl	Pharmacia GST Gene Fusion System Protocol, Second Edition, Revision 2
LB[a, b]	1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5	Maniatis *et al.*, 1982
SOB	2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl	Maniatis *et al.*, 1982
SOC[c]	SOB containing 10 mM $MgCl_2$, 10 mM $MgSO_4$, 0.36% glucose	Maniatis *et al*, 1982
TYP[a]	1.6% tryptone, 1.6% yeast extract, 0.5% NaCl, 0.25% K_2HPO_4	Promega Protocols and Applications Guide, 1989/1990

[a] Ampicillin was added to 100 µg/mL for plasmid selection, as necessary.
[b] For solid media, agar was added to 1.5%.
[c] $MgCl_2$, $MgSO_4$ and glucose were added from stock solutions after autoclaving.

2.2.2 Yeast strains and culture conditions

Yarrowia lipolytica parental strains *E122* and *22301-3* were gifts from Dr. Claude Gaillardin. Genotypes of *Y. lipolytica* parental strains and their derivatives are given in Table 2-4. Construction of derivative strains is discussed in Section 2.11. *Pichia pastoris* strain *PPY3* (*arg4*) and strain *PPY27* (*arg4*, *pex5-1*), also known as *pas8-1*, (Gould *et al.*, 1992; McCollum *et al.*, 1993) were gifts from Dr. Suresh Subramani.

All yeast strains were grown in a rotary shaker at 30°C unless otherwise indicated. Table 2-5 lists the yeast culture media employed in this study.

Table 2-4 ***Y. lipolytica strains used in this study***

Strain	Genotype	Description
E122	*MATA, ura3-302, leu2-270, lys8-11*	Parental strain
22301-3	*MATB, ura 3-302, leu2-270, his1*	Parental strain
pex5-1	*MATA, ura3-302, leu2-270, lys8-11, pex5-1*	Section 2.11.1
pex5-KO	*MATA, ura3-302, leu2-270, lys8-11, pex5::LEU2*	Section 2.11.3
pex5-KOB	*MATB, ura3-302, leu2-270, his1, pex5::LEU2*	Section 2.11.3
DWTAWTB	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, his1/+*	Section 2.11.4
DWTAKOB	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, his1/−, pex5:LEU2/+*	Section 2.11.4
D5AWTB	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/−, his1/−, pex5-1/+*	Section 2.11.4
D5AKOB	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, his1/−, pex5-1/pex5::LEU2*	Section 2.11.4
DKOAWTB	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11/+, his1 −, pex5::LEU2/+*	Section 2.11.4
DKOAKOB	*MATA/MATB, ura3-302/ura3-302, leu2-270/leu2-270, lys8-11 −, his1 −, pex5::LEU2/pex5::LEU2*	Section 2.11.4
E:GDU3	*MATA, ura3-302, leu2-270, lys8-11, PEX5::pGDU3(pex5-G455D,URA3)*	Section 2.11.6
pex5-G455D	*MATA, ura3-302, leu2-270, lys8-11, pex5-G455D*	Section 2.11.6

2.3 Introduction of DNA into microorganisms

2.3.1 Chemical transformation of E. coli

Plasmids were generally amplified in *E. coli* DH5α cells purchased in a transformation-competent state (subcloning efficiency) from Gibco/BRL and transformed

Table 2-5 Yeast culture media

Medium	Composition[a,b]	Reference
2 x CMD	1.34% YNB, CSM (minus leucine and uracil, as required) at twice the manufacturer's recommended concentration, 2% glucose	This study
2 x CMO	1.34% YNB, CSM (minus leucine and uracil, as required) at twice the manufacturer's recommended concentration, 0.05% (w/v) Tween 40, 0.1% (w/v) oleic acid	This study
PSM	0.5% yeast extract, 0.5% ammonium sulphate, 0.2% KH_2PO_4, 2% glucose	Gaillardin *et al.*, 1973
YEPA	2% peptone, 1% yeast extract, 2% sodium acetate	Brade, 1992
YEPD	2% peptone, 1% yeast extract, 2% glucose	Rose *et al.*, 1988
YM	0.5% peptone, 0.3% yeast extract, 0.3% malt extract	Gaillardin *et al.*, 1973
YNA[c]	0.67% YNB, 2% sodium acetate	Brade, 1992
YNA+sorbitol[c]	0.67% YNB, 2% sodium acetate, 1 M sorbitol	Brade, 1992
YND[c]	0.67% YNB, 2% glucose	Rose *et al.*, 1988
YNO[c]	0.67% YNB, 0.05% (w/v) Tween 40, 0.1% (w/v) oleic acid	Nuttley *et al.*, 1993
YPBO	0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 1% Brij 35, 1% (w/v) oleic acid	Kamiryo *et al.*, 1982
SOLT	0.67% YNB, 0.02% (w/v) Tween 20, 0.2% (w/v) oleic acid	Gould *et al.*, 1992

[a] For solid media, agar was added to 2%.
[b] Glucose and oleic acid were added from stock solutions after autoclaving.
[c] Leucine, lysine, histidine and uracil were added to 30 µg/mL, 30 µg/mL, 30 µg/mL and 50 µg/mL, respectively, as necessary.

according to the manufacturer's protocol. A 25 µL aliquot of cells was thawed on ice and mixed with 1-2 µL of a ligation reaction (Section 2.5.8) or 0.5 µL (approximately 0.5 µg) of plasmid DNA. Cells were incubated on ice for 30 min, placed in a 37°C water bath for 20 sec and immediately returned to ice for 2 min. 1 mL of LB was added to the cells, which were then shaken at 250 rpm for 30-45 min at 37°C. Cells were spread onto LB-ampicillin plates and incubated at 37°C overnight to allow for colony formation. If necessary, 75 µL

of 2% X-gal in dimethylformamide was spread on the surface of the agar before the cells were plated to allow for blue/white selection of colonies harbouring recombinant plasmids.

2.3.2 Electroporation

All electroporations used BRL microelectroporation chambers (width ~0.15 cm) in a BRL Cell-Porator connected to a BRL Voltage Booster.

2.3.2.1 Electroporation of *E. coli*

For high-efficiency transformations, plasmids were introduced into *E. coli* by electroporation. Cells were prepared for electroporation essentially by the method suggested by Gibco/BRL. Briefly, 0.5 mL of an overnight culture of *E. coli* in SOB was used to inoculate 500 mL of SOB in a 2 L flask. Cells were grown at 37°C with vigorous aeration until the culture had reached an OD_{600} (optical density at a wavelength of 600 nm) of 0.5 . Cells were harvested by centrifugation at 2,600 x *g* for 10 min at 4°C. The cell pellet was washed twice with ice-cold sterile 10% (v/v) glycerol. The final cell pellet was resuspended in 10% glycerol to a final volume of 2 mL. Cells were either used immediately or frozen as 110 µL aliquots in a dry ice-ethanol bath and stored at -80°C. 1-2 µL of a plasmid-containing solution was gently mixed with 20 µL of cells. Cells were placed between the bosses of a chilled microelectroporation chamber and electroporated with a pulse of 395 V amplified to ~2.4 kV, using a capacitance of 2 µF and a resistance of 4 kΩ. Cells were immediately placed in 1 mL of SOC and grown at 37°C for 1 h in a rotary shaker before plating on LB-ampicillin.

2.3.2.2 Electroporation of *Y. lipolytica*

A 10 mL overnight culture of *Y. lipolytica* cells grown in YEPA was added to 50 mL of fresh YEPA in a 250 mL flask and shaken at 250 rpm at 30°C until an OD_{600} of approximately 1.0 was reached. Cells were harvested by centrifugation at 2,000 x *g* and resuspended in 50 mL of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM lithium acetate. The suspension was gently shaken for 30 min at room temperature or at 30°C. DTT was added to a final concentration of 20 mM, and the incubation was continued for a further 15 min. Cells were collected by centrifugation as before and washed once each in room-temperature water, ice-cold water and ice-cold 1 M sorbitol. Cells were resuspended in the sorbitol solution that remained in the tube after pouring off the final supernatant. 0.5 μL (approximately 0.5 μg) of circular plasmid DNA or 100-300 ng of linearized DNA was gently mixed with 20 μL of cells and placed between the bosses of a chilled microelectroporation chamber. Cells were electroporated with a pulse of 250 V boosted to ~1.6 kV (capacitance 2 μF; resistance 16 kΩ), followed by immediate mixing into 100 μL of 1 M sorbitol and plating onto selective media. When maximum transformation efficiency was required (*e.g.* library screening, integration of linear DNA molecules), the cells were plated on YNA+sorbitol. For routine plasmid transformations, the cells were usually plated on YNA. Colonies were usually visible after 36-48 h of growth at 30°C.

2.3.2.3 Electroporation of *P. pastoris*

The transformation procedure was essentially the same as that for *Y. lipolytica* except that 1) the cells were cultured in YEPD; 2) a resistance of 4 kΩ was used; 3) the

electroporated cells were plated onto selective YND.

2.4 DNA isolation

2.4.1 DNA isolation from bacteria

2.4.1.1 Small-scale plasmid isolations

2.4.1.1.1 Alkaline lysis preparation

The alkaline lysis technique of plasmid isolation was used essentially as described by Maniatis *et al.* (1982). A single bacterial colony was used to inoculate 2 mL of LB-ampicillin. Cells from 1.5 mL of a saturated culture were pelleted by microcentrifugation at 16,000 x *g* for 2 min. The cell pellet was resuspended in 100 µL of 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA. Cellular DNA was denatured by gentle mixing with 200 µL of 0.2 M NaOH, 1% SDS and incubated on ice for 2-3 min. Renaturation of plasmid DNA and precipitation of cellular proteins, high molecular mass RNA and chromosomal DNA were achieved by gentle mixing with 150 µL of potassium acetate solution (3M K^+, 5M acetate), followed by a 5 min incubation on ice. After removal of the precipitate by microcentrifugation for 5 min at 4°C, the supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (26:25:1), followed by an extraction with an equal volume of chloroform/isoamyl alcohol (25:1). Aqueous and organic phases were separated by microcentrifugation at 16,000 x *g* for 2 min. DNA was precipitated from the aqueous phase by mixing with two volumes of absolute ethanol. DNA was pelleted by microcentrifugation for 7 min, and the resultant DNA pellet was rinsed with 1 mL of 70% ethanol and dried in a rotary vacuum desiccator. The dried pellet was dissolved in 40 µL of

TE (pH 8.0) containing 20 µg RNase A/mL. Plasmids were analysed by restriction endonuclease digestion (Section 2.5.1) and, where necessary, by DNA sequencing (Section 2.6.1).

2.4.1.1.2 QIAprep MiniPrep kit

Cells from 1.5 mL of a saturated LB-ampicillin culture were harvested by centrifugation for 2 min at 16,000 x *g*, and the supernatant was removed. Cells were lysed, and plasmid DNA was purified with a QIAprep MiniPrep kit (Qiagen), which uses a modified alkaline lysis procedure to disrupt the cells, followed by purification of the DNA on a silica-gel membrane that selectively adsorbs DNA in the presence of high-salt. The manufacturer's instructions were followed, and the optional wash step with buffer PB was included.

2.4.1.2 Large-scale plasmid isolation

Qiagen DNA purification columns were used according to the manufacturer's specifications when large amounts of purified DNA was required. Cells from 100 mL of a saturated LB-ampicillin culture were harvested by centrifugation at 6,000 x *g* and resuspended in 4 mL of P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg RNase A/mL). After a 5 min incubation on ice, the cellular DNA was denatured by gently mixing in 4 mL of P2 (200 mM NaOH, 1% SDS) and incubating for 5 min at room temperature. To precipitate proteins and chromosomal DNA, 4 mL of P3 (3.0 M potassium acetate, pH 5.5) was added and gently mixed, followed by a 15 min incubation on ice. Precipitated products were separated from the plasmid DNA by centrifugation at 29,000 x *g* for 30 min at 4°C.

The supernatant was further clarified by centrifugation for 15 min at 29,000 x *g* at 4°C and applied to a Qiagen Tip-100 ion-exchange column that had been equilibrated with 10 mL of QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% ethanol, 0.15% (w/v) Triton X-100). The column was washed with 10 mL of QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol), and the DNA was eluted with 5mL of QF (1.25M NaCl, 50 mM Tris-HCl, pH 8.5, 15% ethanol). DNA was precipitated by the addition of 3.5 mL of isopropanol and subjected to centrifugation at 24,500 x *g* for 15 min at 4°C. The DNA pellet was washed with 5 mL of 70% ethanol (v/v) and subjected to centrifugation at 24,500 x *g* for 15 min at 4°C. The DNA pellet was dried in a rotary vacuum desiccator and dissolved in 100 µL TE (pH 8.0).

2.4.2 DNA isolation from yeast

2.4.2.1 Plasmid isolation from yeast

Yeast plasmid DNA was isolated essentially by the method of Ausubel *et al.* (1999). Cells from a 10 mL saturated culture of selective YNA (generally 24-40 h of growth) were harvested by centrifugation at 2,000 x *g* and washed twice in sterile water. Cells were resuspended in 200 µL of yeast nucleic acid disruption buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% (w/v) Triton X-100, 1% SDS) and transferred to a microfuge tube. Glass beads (425-600 µm) were added until they reached the meniscus of the cell suspension. 200 µL of phenol/chloroform/isoamyl alcohol (26:25:1) was added, and the mixture was vortexed vigorously for 5 min at 4°C to disrupt the cells. After the addition of 200 µL of TE (pH 8.0), the organic and aqueous phases were separated by centrifugation at 16,000 x *g* for 5 min at 4°C. The aqueous phase was extracted twice against an equal

volume of phenol/chloroform/isoamyl alcohol (26:25:1) and once against chloroform/isoamyl alcohol (25:1). Nucleic acids were precipitated from the final aqueous phase by the addition of 2.5 volumes of absolute ethanol, followed by incubation at -20°C for 30 min and centrifugation at 16,000 x *g* at 4°C for 45 min. The nucleic acid pellet was rinsed in 1 mL of 70% (v/v) ethanol, dried in a rotary vacuum desiccator and dissolved in 10 µL of TE (pH 8.0). Plasmids were rescued into *E. coli* DH5α by electroporation of 1 µL of the nucleic acid solution (Section 2.3.2.1).

2.4.2.2 Genomic DNA isolation from yeast

The procedure for isolating high molecular mass genomic DNA is identical to that for plasmid DNA isolation (Section 2.4.2.1) except that 1) the cells were grown overnight in YEPD and 2) the nucleic acids were dissolved by placing a 50 µL drop of TE (pH 8.0) containing 20 µg RNase A/mL RNase A on the dried pellet and incubating at 37°C for at least 3 h.

If the genomic DNA were to be used as a template for the polymerase chain reaction (Section 2.5.9), it was further purified by the following method: ammonium acetate was added to the DNA to a final concentration of 100 mM in a total volume of 200 µL. The DNA was extracted twice against an equal volume of phenol/chloroform/isoamyl alcohol (26:25:1) and once against an equal volume of chloroform/isoamyl alcohol (25:1). DNA was precipitated from the aqueous phase by the addition of 2.5 volumes of absolute ethanol followed by centrifugation at 16,000 x *g* for 10 min. The DNA pellet was rinsed with 1 mL of 70% (v/v) ethanol and dried in a rotary vacuum desiccator. The DNA pellet was

resuspended in 50 µL of TE (pH 8.0) and dissolved by incibation at 37°C for at least 3 h.

2.5 Standard DNA manipulations

Unless otherwise noted, all procedures in this section are essentially as reported by Ausubel *et al.* (1999).

2.5.1 Restriction endonuclease digestion

Typically, 1-2 µg of plasmid DNA or 10 µg of genomic DNA was subjected to restriction endonuclease digestion. Digestion conditions varied but were carried out with consideration for the enzyme manufacturers' suggestions using either a supplied buffer or a dilution of the KGB buffer system (Hanish and McClelland, 1988).

2.5.2 Dephosphorylation of 5' ends

Phosphate groups were often removed from the 5'-termini of plasmid vectors to prevent self-ligation. To a 20 µL restriction endonuclease digest, 1 unit of calf intestinal alkaline phosphatase (CIP) was added, and the reaction was incubated at 37°C for 30-45 min. When necessary, reactions were terminated by heating at 75°C for 15 min.

2.5.3 Phosphorylation of 5' ends

Occasionally, it was necessary to phosphorylate the 5'-termini of DNA molecules to render them suitable for ligation. For phosphorylation of PCR products (Section 2.5.9), 23 µL of a PCR reaction was brought to a final volume of 30 µL by the addition of 3 µL of 10

x PNK buffer (700 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT), 3μL of 10 mM ATP and 1 μL (10 U) of T4 polynucleotide kinase. The reaction was allowed to proceed at 37°C for 1 h.

2.5.4 Creating blunt-ended fragments

2.5.4.1 Polishing 5' overhangs

The appropriate restriction endonuclease digestion was performed in a volume of 20 μL. To this was added 5 U of the Klenow fragment of *E. coli* DNA polymerase I and deoxyribonucleoside triphosphates (dNTPs) to a final concentration of 100 μM (*i.e.* 25 μM each dNTP) in a total volume of 25 μL. The reaction was allowed to proceed at 30°C for 15 min. The reaction was usually terminated by immediately subjecting the reaction to agarose gel electrophoresis (Section 2.5.7) or, less commonly, by heating to 75°C for 15 min or extraction with phenol/chloroform (Section 2.5.5).

2.5.4.2 Polishing 3' overhangs

The appropriate restriction endonuclease digestion was carried out in a volume of 20 μL. To this was added 9 U of T4 DNA polymerase and dNTPs to a final concentration of 100 μM (*i.e.* 25 μM each dNTP) in a total volume of 25 μL. The reaction was allowed to proceed at 11°C for 20 min. The reaction was terminated by heating at 75°C for 15 min.

2.5.5 Phenol/chloroform extraction

Extraction in phenol/chloroform was sometimes used to terminate enzymatic reactions

and/or to separate DNA from contaminating protein. DNA was brought to a volume of 200 µL by the addition of water. An equal volume of phenol/chloroform/isoamyl alcohol (26:25:1) was added, and the sample was vortexed vigorously for 15 sec. After phase separation by centrifugation at 16,000 x *g* for 2 min at room temperature, the aqueous phase was removed to a fresh microfuge tube. The extraction was repeated using an equal volume of chloroform/isoamyl alcohol (25:1) and the aqueous phase was again removed to a fresh tube. DNA was concentrated by precipitation with ethanol (Section 2.5.6)

2.5.6 Ethanol precipitation of DNA

To a dilute solution of DNA in a low-salt buffer (less than 100 mM), 25 µg of linear polyacrylamide prepared by the method of Gaillard and Strauss (1990) was added, and the mixture was brought to a final concentration of 0.3 M sodium acetate, pH 5.2. To this, 2.5 volumes of -20°C absolute ethanol was added, and the tube was briefly vortexed. Precipitation was allowed to occur at -80°C for at least 15 min or -20°C for at least 30 min. Precipitated products were collected by centrifugation at 16,000 x *g* for 45 min at 4°C. The pellet was rinsed with 70% (v/v) ethanol and dried in a rotary vacuum desiccator. The dried pellet was dissolved in water or a solution appropriate for subsequent reactions.

2.5.7 Agarose gel electrophoresis of DNA fragments

After the appropriate enzymatic manipulations, 0.2 volume of 6 x DNA sample dye (40 % sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol) (Maniatis *et al.*, 1982) was added to the reaction mixture. DNA fragments were separated by electrophoresis in agarose

gels (typically 0.8-1.5%) in 1 x TBE containing 0.5 μg ethidium bromide/mL. For analytical gels, Ultra Pure grade agarose (Gibco/BRL) was used. For preparative gels, SeaKem Genetic Technology Grade (GTG) agarose (FMC BioProducts) was used. For resolution of fragments less than 400 bp in length, 3% "voodoo agarose" gels were used, consisting of 0.5% SeaKem GTG agarose and 2.5% NuSieve GTG agarose. The gels were viewed on an ultra-violet transilluminator (Photodyne, Model 3-3006).

2.5.7.1 Purification of DNA fragments

Restriction endonuclease-digested DNA was subjected to electrophoresis in gels made from GTG agarose. Slices of the gel containing the appropriate DNA fragments were excised with a razor blade. Recovery of DNA from gel slices after purification was assessed by the electrophoresis of 1 μL of the final product in an analytical agarose gel and visually comparing its staining by ethidium bromide to that of a known amount of DNA molecular size standards.

2.5.7.1.1 Electroelution

A gel slice was placed in the well of a unidirectional electroeluter (Model UEA, International Biotechnologies) filled with 0.5 x TBE. 80 μL of a high molar salt solution (7.5 M ammonium acetate, 0.25% bromophenol blue) was carefully deposited into the bottom of the sample tunnel connecting the two reservoirs of the apparatus. A current of 100 mV was applied for 30 min. Eluted DNA was collected along with the high molar salt by taking 350 μL of liquid from the bottom of the tunnel. 25 μg of linear polyacrylamide (Gaillard and

Strauss, 1990) and 1 mL of absolute ethanol chilled to -20°C were added, and the DNA was precipitated by incubation at -80°C for 15 min. The precipitate was collected by centrifugation at 16,000 x *g* for 45 min at 4°C. The pellet was washed in 1 mL of 70% (v/v) ethanol, dried in a rotary vacuum desiccator and dissolved in 10 µL of water.

2.5.7.1.2 QIAquick columns

QIAquick columns (Qiagen) were used according to the manufacturer's instructions for DNA isolation using a microcentrifuge. This technology employs a chaotropic salt solution to dissolve the agarose gel slice, after which the DNA is purified on an ion exchange resin. DNA was eluted from the column by the addition of 30 µL of 10 mM Tris-HCl, pH 8.5.

2.5.8 Ligation of DNA fragments

DNA fragments were combined at an insert:vector molar ratio of 5:1 with a maximum total DNA concentration of 20 ng/µL. Ligation reactions were generally performed in a total volume of 10 µL, using 1 U of T4 DNA ligase in the manufacturer's supplied buffer and a final ATP concentration of 1 mM. Ligations involving blunt-ended fragments were always incubated overnight at 16°C. Ligations involving fragments with cohesive overhangs were either incubated overnight at 16 °C or at room temperature for more than 2 h. Ligation products were amplified by transformation of *E. coli* (Section 2.3.1).

2.5.9 Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify specific DNA sequences to facilitate cloning or to introduce modifications in the amplified sequence. PCR was performed with either a conventional preparation of *Taq* polymerase (Roche) using established procedures (Innis and Gelfand, 1990; Saiki, 1990) or with Ready-to-Go PCR beads (Amersham-Pharmacia). Template DNA was either 0.5 µg of the appropriate plasmid or 1 µg of *Y. lipolytica* genomic DNA prepared as described in Section 2.4.2.2. PCR reactions were carried out in either a Techne PHC-2 thermocycler connected to a Neslab Endocal refrigerated circulating water bath or in a Robocycler 40 (Stratagene) with or without a Hot Top.

2.5.9.1 *Taq* Polymerase

Reactions were carried out in a total volume of 100 µL including template DNA, the supplied reaction buffer, 5 U of *Taq* polymerase (Roche), 20, 50 or 100 pmol of each specific primer and 50 µM each of dATP, dCTP, dGTP and dTTP. The reaction mix was overlaid with 75 µL of mineral oil to prevent evaporation. After completion of thermocycling, the aqueous phase was separated from the mineral oil by spotting repeatedly onto Parafilm.

2.5.9.2 Ready-to-Go PCR beads

Reactions were performed in a total volume of 25 µL and consisted of template DNA, 25 pmol of each specific primer and one Ready-to-Go™ PCR bead (containing reaction buffer components, dNTPs and *Taq* polymerase). If the PCR machine to be used was not equipped with a Hot Top, the reaction mix was overlaid with 30 µL of mineral oil to prevent

evaporation. After completion of thermocycling, the aqueous phase was separated from the mineral oil by spotting onto Parafilm.

2.5.9.3 PCR product purification

If the PCR product was to be used as a substrate for a further PCR step or for restriction endonuclease digestion, the reaction mixture was brought to 100 µL with water, and DNA was purified on a QIAquick column (Qiagen) according to the manufacturer's instructions. The kit employs a chaotropic agent to inactivate the *Taq* polymerase, and an ion exchange column that binds DNA fragments but excludes primers smaller than 40 nucleotides in length. The column was eluted with 30 µL of elution buffer (10 mM Tris-HCl, pH 8.5).

2.6 Analyses of DNA

2.6.1 DNA sequencing

2.6.1.1 Template preparation

Denatured plasmids were used as templates for sequencing reactions (Zhang *et al.*, 1988). Approximately 3 µg of double-stranded plasmid DNA was brought to 0.5 mM EDTA and 500 mM NaOH in a volume of 18 µL. After allowing the plasmid to denature for 5 min at room temperature, the solution was neutralised by addition of ammonium acetate to 500 mM in a final volume of 20 µL. The DNA was immediately precipitated by addition of 3 volumes of ethanol chilled to -20°C and incubation in a dry ice/ethanol bath or at -80°C for at least 20 min. Precipitated DNA was collected by centrifugation at 16,000 x *g* for 45 min

at 4°C. The DNA pellet was rinsed with 70% (v/v) ethanol and dried in a rotary vacuum desiccator. Templates were either used immediately or stored at -20°C.

2.6.1.2 Sequencing reactions

Sequenase DNA Sequencing Kits (Versions 1.0 and 2.0; USB) were used. These kits generate sequences by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977), use α-[^{32}P]dATP as the label and employ bacteriophage T7 DNA polymerase that has been chemically or genetically modified (Sequenase Versions 1.0 and 2.0, respectively) (Tabor and Richardson, 1987; 1989) to remove the 3'→5' exonuclease activity of the wild-type enzyme. The manufacturer's instructions were followed, except that twice as much specific primer was used (*i.e.* 2 pmol). Labelling and termination reactions were incubated for 5 min each. Reaction products were run in adjacent lanes of a denaturing acrylamide gel (5% Long Ranger or 5% ExplorER, in 0.6 x TBE or 1 x TBE, respectively). The gel was dried and exposed to X-ray film at room temperature for detection.

2.6.2 Analysis of genomic DNA

2.6.2.1 Southern blotting

Following transformation with linear DNA fragments (Section 2.3.2.2) or after selection against unstable DNA configurations (Section 2.11.6), genomic DNA was analysed to identify strains that had recombined in a site-specific manner. Genomic DNA was obtained from yeast cells as described in Section 2.4.2.2. Approximately 10 μg of DNA was digested overnight with a restriction endonuclease(s) (Section 2.5.1). Digestion products were

separated by agarose gel electrophoresis (Section 2.5.7). DNA was transferred to nitrocellulose essentially by the method of Ausubel *et al.* (1999), as modified by Eitzen (1997). The gel was placed on an ultra-violet light transilluminator (Photodyne, Model 3-3006) for 10 min to nick the DNA. Complementary DNA strands were denatured by gently shaking the gel in 0.5 M NaOH, 1.5 M NaCl for 30 min at room temperature. The gel was neutralised by gently shaking in 1.5 M Tris-HCl, pH 8.0, 1.5 M NaCl for 30 min at room temperature. Nitrocellulose was hydrated in water for 5 min, and then equilibrated in 5 x SSC. DNA was transferred from the gel to nitrocellulose in 5 x SSC by capillary action overnight. DNA was crosslinked to the nitrocellulose by exposure to ultra-violet light (twice at 120,000 $\mu J/cm^2$, $\lambda = 254$ nm; UV Stratalinker 1800, Stratagene). Immobilized DNA was probed for complementary sequences by hybridization of labelled DNA fragments (see below).

2.6.2.2 Labelling and hybridization of DNA probes

2.6.2.2.1 Radiolabelled probes

DNA probes were labelled with ^{32}P using the Random Primer DNA Labeling Kit (Roche). The DNA fragment to be used as a probe was excised from an agarose gel and purified as described in Section 2.5.7.1. 25-50 ng of DNA was brought to 9 µL with water, denatured by boiling for 5 min and rapidly cooled in ice-water for 5 min. 2 µL of reaction mixture (containing random hexanucleotides), 1 µL each of 0.5 mM dGTP, dCTP and dTTP, 5 µL (50 µCi) of α-[^{32}P]dATP (3,000 Ci/mmol) and 1 µL (5 U) of the Klenow fragment of DNA polymerase I were added. The mixture was incubated at 37°C for 30 min. The labelled

probe was separated from unincorporated radionucleotides by Sephadex G-50 spin column chromatography (Maniatis *et al.*, 1982). Radionucleotide incorporation was assessed by liquid scintillation counting in an LKB RackBeta 1209 scintillation counter. Radiolabelled probes were either used immediately or stored at -20°C.

After the transfer of genomic DNA to nitrocellulose, the unoccupied DNA binding sites on the nitrocellulose were blocked by incubating the blot with hybridization solution (1.25 x SSC, 0.16 x Denhardt's solution, 4 µg sheared salmon testes DNA/mL, 0.01% SDS, 0.02 M sodium phosphate, pH 7.0) to approximately 0.25 mL hybridization solution per cm^2 of nitrocellulose for 3 h at 65°C. Enough radiolabelled probe to give a final concentration of 500,000 cpm/mL during hybridization was denatured by boiling for 5 min, quick-cooled in ice-water for 5 min and diluted into 1 mL of hybridization solution pre-warmed to 42°C. The probe solution was added to the buffer used to block the blot and incubation was continued overnight at 42°C. The blot was then washed four times with 1 x SSC containing 0.1% SDS for 15 min at 55°C. The blot was dried, covered with Saran wrap and exposed to Kodak XAR-5 X-ray film with an intensifying screen at -80°C.

2.6.2.2.2 Chemiluminescent probes

DNA probes were labelled with horseradish peroxidase (HRP) using the enhanced chemiluminescence (ECL) direct nucleic acid labelling system (Amersham-Pharmacia). The DNA probe fragment was excised from an agarose gel and purified as described in Section 2.5.7.1. HRP-labelled probes were freshly prepared according to the manufacturer's instructions, using 2.5 ng of DNA per cm^2 of nitrocellulose to be probed.

The hybridization procedure suggested by the manufacturer was used. Unoccupied DNA binding sites on the blot were blocked by incubation with Gold blocking buffer (Gold buffer containing 0.5 M NaCl, 5% blocking agent) at approximately 0.25 mL per cm^2 of nitrocellulose at 42°C for at least 1 h. Freshly prepared HRP-labelled probe was diluted into 1 mL of prewarmed Gold blocking buffer and mixed with the buffer that had been used to block the blot. Incubation was continued at 42°C overnight. The blot was washed twice in primary wash buffer (0.5 x SSC, 0.4% SDS, 6 M urea) at approximately 2 mL per cm^2 of nitrocellulose) at 42°C for 20 min per wash. The blot was then washed twice in 2 x SSC for 5 min at room temperature. The blot was covered with ECL detection solution (a 1:1 mixture of the two ECL detection reagents) for 1 min, placed in a transparent plastic folder and exposed to Kodak XK-1 film.

2.7 *In vitro* mutagenesis

Site-directed mutagenesis was carried out on single-stranded DNA templates using the Sculptor *In Vitro* Mutagenesis Kit (Amersham-Pharmacia) essentially as described by the manufacturer.

2.7.1 Transformation of E. coli TG1

0.25 mL of an overnight culture of *E. coli* TG1 cells grown in LB-ampicillin was used to inoculate 5 mL of fresh medium, which was shaken at 37°C until an OD_{600} of 0.3 was reached. Cells were harvested by centrifugation at 3,000 x *g* for 5 min and resuspended in 5 mL of ice-cold 50 mM $CaCl_2$. After a 20 min incubation on ice, the cells were pelleted by

centrifugation at 3,000 x *g* for 2 min and resuspended in 1 mL of ice-cold $CaCl_2$. 1-10 ng of the appropriate plasmid was brought to 20 μL with TE (pH 8.0). 0.3 mL of the cell suspension was mixed with the diluted plasmid and incubated on ice for 40 min. Cells were incubated at 42°C for 2 min and then returned to ice for a further 5 min. 1 mL of LB was added, and the cells were shaken at 37°C for 45-60 min. Cells were spread onto an LB-ampicillin plate and incubated overnight at 37°C to allow for colony formation. Resulting colonies were stored for no more than a few hours at 4°C before growing in liquid medium for single-stranded DNA isolation.

2.7.2 Single-stranded DNA preparation

Single stranded DNA was isolated essentially as described in the Promega Protocols and Applications Guide (1989/90). A single TG1 transformant colony was inoculated into 2 mL of TYP-ampicillin and shaken overnight at 37°C. 1 mL of the overnight culture was subcultured into 50 mL of fresh TYP-ampicillin and shaken for 30 min at 37°C. Cells were then infected with the helper phage M13KO7 at a multiplicity of infection of 10-20, and shaking was continued at 37°C for 7 h. The culture was subjected to centrifugation at 12,000 x *g* for 15 min to pellet bacteria and cell debris. The supernatant containing the phage particles was retained and subjected to centrifugation as before. The second supernatant was brought to 10 μg RNase A/mL and incubated at 37°C for 15 min. Phage particles were precipitated by the addition of 0.25 volume of phage precipitation buffer (3.75 M ammonium acetate, pH 7.5, 20% PEG-8000) and incubation on ice for 30 min. The precipitate was pelleted by centrifugation at 12,000 x *g* for 15 min at 4°C. The phage pellet was resuspended

in 600 µL of TE (pH 8.0). An equal volume of chloroform/isoamyl alcohol (25:1) was added and the phage particles were disrupted by vortexing at high speed for 1 min. Aqueous and organic phases were separated by centrifugation at 12,000 x *g* for 5 min. The aqueous phase containing the single-stranded DNA was extracted against an equal volume of phenol/chloroform/isoamyl alcohol (26:25:1) by vortexing and centrifugation as before. Extraction with phenol/chloroform/isoamyl alcohol was repeated several times until there was no material visible at the interface between the aqueous and organic phases. The aqueous phase was then extracted with an equal volume of chloroform/isoamyl alcohol. DNA was precipitated from the final aqueous phase by the addition of 0.5 volume of 7.5 M ammonium acetate and 2 volumes of absolute ethanol chilled to -20°C. The mixture was incubated at -20°C for 30 min, and the DNA was recovered by centrifugation at 16,000 x *g* for 30 min. The pellet was washed with 1 mL of 70% (v/v) ethanol and subjected to centrifugation at 16,000 x *g* for 15 min. The pellet was dried in a rotary vacuum desiccator and dissolved in 20 µL of water.

2.7.3 Phosphorylation of mutagenic oligonucleotides

A reaction mixture containing 1.6 µM oligonucleotide, 1 mM ATP, 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT and 2 U of T4 polynucleotide kinase in a final volume of 30 µL was incubated at 37°C for 15 min. The kinase was inactivated by a 10 min incubation at 75°C.

2.7.4 Mutagenesis reactions

In vitro, site-directed mutagenesis was carried out according to the manufacturer's instructions with the following modifications: 1) to anneal the mutant oligonucleotide to the single-stranded DNA template, the tube was placed in a 70°C heat block for 3 min, after which the block was removed from the heater and allowed to cool slowly to room temperature; 2) to extend the mutant oligonucleotide, 4 units of the Klenow fragment of DNA polymerase I were used. The extension/ligation reaction was incubated at 16°C overnight; 3) the final extension/ligation reaction to repolymerize the gapped DNA was incubated at 37°C for 90 min, followed by a 30 min incubation at 16°C. 10 μL of this reaction was removed for a gel sample; 4) 1 μL of the repolymerization reaction was added to 9 μL of TE (pH 8.0), and 1 μL of the resulting mixture was introduced by electroporation into *E. coli* DH5α cells (Section 2.3.2.1). DNA from the remainder of the repolymerization reaction was concentrated by a method suggested in the protocol accompanying a previous version of the mutagenesis kit (Oligonucleotide-directed Mutagenesis System Version 2). The remaining 85 μL of the repolymerization reaction was mixed with 85 μL of 4 M ammonium acetate (pH 5.4) and 340 μL of absolute ethanol to precipitate the DNA. After a 10 min incubation at room temperature, the DNA was subjected to centrifugation at 16,000 x *g* for 15 min. The pellet was washed with 1 mL of 70% (v/v) ethanol and repelleted by centrifugation. The pellet was dried in a rotary vacuum desiccator and dissolved in 10 μL of TE (pH 8.0). 1 μL and 2 μL samples of the final solution were introduced by electroporation into *E. coli* DH5α cells.

2.8 Analyses of proteins

2.8.1 Protein determination

The protein concentration of a sample was determined essentially by the method of Bradford (1976). A portion of the protein sample was brought to a final volume of 100 μL with water. 1 mL of Bio-Rad Protein Dye reagent was added, mixed by vortexing and incubated at room temperature for 10 min to allow for colour development. The absorbance at 595 nm of the resulting solution was measured using a Beckman DU640 spectrophotometer. Protein concentration was determined by comparing the absorbance of the sample to that of known amounts of BSA (1, 2, 5, 10, 15, 20, 25 μg) treated identically.

2.8.2 Electrophoretic separation of proteins

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) essentially by the method of Laemmli (1970). Protein samples were mixed with concentrated sample buffer to a final concentration of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% sucrose, 10 mM DTT, 0.001% bromophenol blue and denatured by boiling for 5 min. Samples were subjected to electrophoresis on discontinuous slab gels, usually with a uniform acrylamide concentration in the resolving gel. Stacking gels consisted of 3% acrylamide (30:0.8 acrylamide:*N,N'*-methylene-bis-acrylamide), 60 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.1% (v/v) TEMED, 0.1% ammonium persulphate. Resolving gels typically consisted of between 7.5% and 15% acrylamide (30:0.8 acrylamide:N,N'-methylene-bis acrylamide), 370 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.1% (v/v) TEMED, 0.042% ammonium persulphate. Occasionally, to resolve proteins over a wide size range, the resolving gel was made with a gradient of acrylamide (8-15%). Gels were run in either a Hoefer Model

SE4300 or a Bio-Rad Mini-Protean II vertical gel system at 50-200 V in SDS-PAGE running buffer (50 mM Tris-HCl, pH 8.8, 0.4 M glycine, 0.1% SDS).

2.8.3 Detection of proteins

2.8.3.1 Staining of SDS-polyacrylamide gels with Coomassie Blue

Proteins were visualised by staining gels with 0.1% Coomassie Brilliant Blue (R-250) in 10% (v/v) acetic acid, 35% (v/v) methanol for at least 1 h with gentle agitation. Gels were destained by multiple washes in 10% (v/v) acetic acid, 35% (v/v) methanol. Stained gels were either processed further (see below) or dried at 80°C on a Bio-Rad gel dryer (Model 583).

2.8.3.2 Fluorography

SDS-polyacrylamide gels containing ^{35}S-labelled proteins were processed for fluorography (Bonner and Laskey, 1974). Gels were stained and destained as described in Section 2.8.3.1. and then dehydrated by two 15 min washes in dimethylsulphoxide (DMSO). Gels were incubated for 3 h in DMSO-PPO (22.2% 2,5-diphenyloxazole in DMSO). Gels were rehydrated with two 10 min washes in water, dried at 60°C and exposed to preflashed Kodak XAR-5 X-ray film at -80°C.

2.8.3.3 Immunoblotting

Nitrocellulose was hydrated in water for 5 min and then equilibrated in Western transfer buffer (20 mM Tris base, 150 mM glycine, 20% (v/v) methanol) (Towbin, 1979;

Burnette, 1981). Proteins separated by SDS-PAGE were transferred to nitrocellulose in Western transfer buffer, using either a wet or semi-dry apparatus. For wet blotting, the transfer was carried out at 100 mA for approximately 16 h at room temperature, or at 400 mA forapproximately 4 h using a cooling coil, in a blotting tank (Bio-Rad Trans-Blot or Hoefer TE Series Transphor electrophoresis unit). For semi-dry blotting, the transfer was carried out at 0.8 mA per cm^2 of gel in an ET-20 electrophoretic transfer system (Tyler Research Instruments, Edmonton, Alberta). Transferred proteins were visualised by immersing the nitrocellulose blot in Ponceau stain (0.1% Ponceau S in 1% trichloroacetic acid) for 2-3 min and destaining with water. Unoccupied protein binding sites on the nitrocellulose were blocked by incubating the blot in TBST-milk (1% skim milk powder in TBST; see Table 2-2) for 30 min with gentle agitation. Primary antiserum was diluted in TBST-milk at the concentrations indicated in Table 2-6. The blots were incubated with the primary antiserum for 90 min at room temperature. Excess primary antibody was removed by four 10 min washes in TBST. The appropriate secondary antibody conjugated to HRP was diluted in TBST-milk at a concentration of 1:30,000 and incubated with the blots for 30 min. Excess secondary antibody was removed by four 10 min washes in TBST. The blot was covered with ECL detection solution (a 1:1 mixture of the two ECL detection reagents) for 1 min, placed in a transparent plastic folder and exposed to Kodak XK-1 X-ray film. For quantitation of signals, densitometry was performed with a LKB Ultroscan XL laser densitometer under conditions in which the signal was proportional to the antigen concentration.

Table 2-6 Primary antibodies used for immunoblotting[a]

Target	Source	Concentration
*Sc*AOX	rabbit	1:500
*Sc*G6PDH	rabbit	1:20,000
*Yl*Kar2p	rabbit	1:20,000
*Pp*Pex5p	rabbit	1:3,000
*Yl*Pex5p	rabbit Q	1:1,000
*Yl*Pex5p	guinea pig N	1:50,000
SKL	rabbit 16	1:1,000
thiolase	guinea pig N	1:50,000

[a] Antibodies are described in Section 2.1.7.

2.8.4 Precipitation of proteins

Dilute solutions of proteins were concentrated by precipitation with trichloracetic acid (TCA). TCA was added to the protein solution to a final concentration of 10%. The mixture was incubated on ice for 30 min and then subjected to centrifugation at 16,000 x *g* for 30 min at 4°C. The pellet was washed twice with 1 mL of 80% (v/v) acetone chilled to -20°C. For each wash, the tube was vortexed and then incubated on ice for 10 min before microcentrifugation at 16,000 x *g* for 10 min at 4°C. The final pellet was dried in a rotary vacuum desiccator, resuspended in SDS-PAGE sample buffer and analysed by SDS-PAGE and Western blotting or staining with Coomassie Blue.

2.9 *In vitro* translation

2.9.1 Preparation of RNase-free template DNA

To ensure that DNA templates for *in vitro* transcription were free of contaminating RNase, 20 μg of plasmid DNA purified on Qiagen columns (Section 2.4.1.2) was extracted twice with phenol/chloroform/isoamyl alcohol and twice with chloroform/isoamyl alcohol (Section 2.5.5), and then precipitated with ethanol (Section 2.5.6). The DNA pellet was rinsed with 1 mL of 70% (v/v) ethanol prepared with diethyl pyrocarbonate (DEPC)-treated water. The pellet was dried in a rotary vacuum desiccator and dissolved in 20 μL of DEPC-treated water (Ausubel *et al.*, 1999).

2.9.2 Coupled transcription/translation

In vitro transcription and translation were carried out using the TNT coupled reticulocyte lysate system with T7 RNA polymerase (Promega) according to the supplier's instructions. 0.5 μg of plasmid template was used in a 25 μL reaction. For synthesis of radiolabelled proteins, 10 μCi of [^{35}S]methionine (1175 Ci/mmol, 10 mCi/mL) was used.

2.9.3 Quantitation of radiolabelled translation products

Incorporation of [^{35}S]methionine into polypeptides was assessed essentially by the method of Mans and Novelli (1961). 2.5 μL of each transcription/translation reaction was mixed with 47.5 μL of NP-40 mix (10 mM Tris-HCl, pH 8.5, 150 mM NaCl, 200 mM methionine, 0.02% sodium azide, 1% (w/v) Nonidet P-40 (NP-40)). Duplicate 20 μL samples were spotted on 1 cm^2 squares of filter paper and allowed to air dry. The spotted filters, plus a blank filter control, were placed in 100 mL of ice-cold 10% TCA containing 200 mM methionine and incubated on ice for 10 min with occasional stirring. The filters

were then transferred to 100 mL of boiling 5% TCA containing 200 mM methionine and boiling was continued for 20 min. The filters were then washed four times in 5% TCA, twice in absolute ethanol, and once in diethyl ether. Each wash used 50 mL of solution and was carried out at room temperature for 30-60 sec with stirring. The filters were then air-dried, placed in 5 mL of aqueous counting scintillant, and the precipitated radioactivity was quantitated in an LKB RackBeta 1209 scintillation counter.

2.10 Cell Biological Techniques

2.10.1 Immunofluorescence microscopy

Indirect immunofluorescence analysis of yeast cells was performed by the method of Pringle *et al.* (1991). Log phase cells were fixed by adding formaldehyde directly to the culture medium to a final concentration of 3.7% and incubating for 45 min at room temperature. For experiments involving anti-*Yl*Pex5p antibodies, the cells were collected by centrifugation and incubated for 2 h in phosphate-buffered formaldehyde (50 mM potassium phosphate, pH 6.5, 0.5 mM $MgCl_2$, 3.7% (w/v) formaldehyde). Fixed cells were harvested by centrifugation at 2,000 x *g* and washed with solution B (100 mM potassium phosphate, pH 7.5, 1.2 M sorbitol). Cells were resuspended to a concentration of 50-100 µg/mL in solution B containing 20 µg/mL Zymolyase 100T and 28 mM 2-mercaptoethanol, and incubated at 30°C for 45 min with gentle rotation. During this time, a 50 µL-drop of a 1 mg polylysine/mL solution was applied to a glass microscope slide and allowed to remain for approximately 30 sec. Excess liquid was then removed and the slide was dried in air. 100 µL of the cell suspension was placed on the polylysine spot, allowed to remain for

approximately 30 sec, and then washed with solution B until individual cells were visibly separated when viewed with a light microscope. After complete drying in air, the slides were submerged in methanol (chilled to -20°C) for 6 min, immediately transferred to acetone (chilled to -20°C) for 30 sec and then allowed to dry in air. Cells were covered with a 75-µL drop of PBS (Table 2-2) containing 1% skim milk powder (PBS-milk) and the appropriate dilution of primary antiserum (generally 10 times more concentrated than that used for immunoblotting; Section 2.8.3.3). Slides were incubated for 1 h at room temperature in a moist environment. Cells were washed 10 times with 100 µL of PBS-milk, and then covered with 75 µL of PBS-milk containing the appropriate secondary antibody conjugated to fluorescein or rhodamine at a dilution suggested by the manufacturer (generally 1:200). Cells were incubated in darkness for 1 h at room temperature in a moist environment and then washed as described above. The last wash was replaced with 10 µL mounting medium (PBS containing 4% n-propyl gallate, 75% (w/v) glycerol). Coverslips were placed over the cells, and the edges were sealed with nail polish. Cells were viewed on an Olympus BX50 microscope equipped for fluorescence. Images were either photographed with an Olympus PM20 automated camera and Kodak TMAX400 film or recorded using a SPOT Camera (Model SP400, SPOT Diagnostic Instruments) digital imaging system and analysed with software provided by the manufacturer.

2.10.2 Electron microscopy

All experiments of this type were performed by Dr. Marten Veenhuis, Laboratory for Electron Microscopy, University of Groningen, The Netherlands, using established methods

(Waterham *et al.*, 1992).

2.10.2.1 Ultrastructural analysis

Cells were fixed in 1.5% $KMnO_4$ for 20 min at room temperature. After dehydration in a series of graded ethanol, samples were embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Phillips EM 300.

2.10.2.2 Immunocytochemistry

Cells were fixed in 3% (v/v) glutaraldehyde/formaldehyde in 0.1 M sodium cacodylate (pH 7.2) for 90 min at 4°C. After dehydration in a series of graded ethanol, the cells were embedded in Lowicryl K4M. Ultrathin sections were probed for thiolase and anti-SKL reactive proteins by passage through drops of the following solutions at room temperature for the times indicated: 1) 2% gelatin in PBS for 10 min; 2) 0.02 M glycine in PBS for 10 min; 3) rabbit anti-SKL or guinea pig anti-thiolase antibodies diluted in PBS for 30 min; 4) four washes in PBS for 1 min each; 5) protein A-gold in PBS containing 1% BSA for 30 min; 6) 4 washes in PBS for 5 min each (Slot and Geuze, 1984).

2.10.3 Whole cell lysates of yeasts

Yeast lysates were prepared by disruption with glass beads (adapted from Needleman and Tzagoloff, 1975). Log phase yeast grown in the appropriate medium were harvested by centrifugation at 2000 x *g* and washed three times with water. Cells were resuspended in an equal volume of ice-cold breakage buffer (either 25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA,

100 mM KCl, 1 mM DTT, 10% (w/v) glycerol (Eitzen *et al.*, 1997) or 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10% (w/v) glycerol) containing protease inhibitors at the following final concentrations: leupeptin, pepstatin and aprotinin each at 1 μg/mL, 0.5 mM benzamidine hydrochloride, 5 mM NaF, 1 mM PMSF. Glass beads were added to 200 μL of the cell suspension until they reached the bottom of the meniscus. Cells were disrupted by vortexing 3-5 times for 1 min each (with 1 min on ice in between) at 4°C. A further 100-200 μL of breakage buffer was added and mixed by briefly vortexing. The mixture was subjected to centrifugation at 16,000 x *g* for 2 min at 4°C to separate the lysate from the glass beads and large cell debris. The crude lysate was recovered to a new tube and subjected to centrifugation at 16,000 x *g* for 20 min at 4°C. The supernatant was recovered, assayed for protein content (Section 2.8.1) and usually analysed by SDS-PAGE and immunoblotting (Sections 2.8.2 and 2.8.3.3, respectively).

2.10.4 Peroxisome isolation and analysis

All experiments of this type were performed by Dr. Vladimir Titorenko.

2.10.4.1 Subcellular fractionation

Y. lipolytica cells were fractionated into supernatant (20KgS; enriched for cytosol) and pellet (20KgP; enriched for peroxisomes and mitochondria) fractions essentially as described previously (Aitchison *et al.*, 1991). Cells were grown to mid-log phase in YEPD, harvested by centrifugation at 9800 x *g* and transferred to YPBO to induce peroxisome formation. Cells were harvested in mid-log phase (approximately 9 h of induction) as above

and washed three times with water at room temperature. Cells were converted to spheroplasts by resuspending the cells in 0.5 M KCl, 5 mM MOPS, pH 7.2, 10 mM sodium sulphite, 0.25 mg Zymolyase 100T/mL (4 mL per g of cells) and incubating at 30°C with gentle agitation (100 rpm) for 30 min. Spheroplasts were pelleted by centrifugation at 2,300 x *g* for 8 min at 4°C. From this point on, all solutions were ice-cold, and all centrifugations were performed at 4°C. Spheroplasts were resuspended in disruption buffer (5 mM MES, pH 5.5, 1 M sorbitol, 0.5 mM EDTA, 1 mM KCl, 0.1% (v/v) ethanol plus protease inhibitors as described in Section 2.10.3; 3 mL per g of cells), transferred to a homogenization tube, and broken with 20 strokes of a Teflon pestle driven at 1,000 rpm by a stirrer motor (Cole-Parmer, Model 4376-00). The homogenate was subjected to centrifugation at 1,000 x *g* for 10 min to pellet unbroken cells, debris and nuclei. The post-nuclear supernatant was subjected to centrifugation for 30 min at 20,000 x *g* to generate a pellet (20KgP) enriched for heavy organelles (mostly peroxisomes, and mitochondria) and a supernatant (20KgS) enriched for cytosol. The 20KgP was gently resuspended in approximately 3 mL of disruption buffer.

2.10.4.2 Isolation of organelles by isopycnic centrifugation

Organelles from the 20KgP were separated by isopycnic centrifugation by loading an amount of 20KgP equivalent to 9 mg of protein on top of a discontinuous sucrose gradient (4.7 mL 25%, 7 mL 35%, 14 mL 42%, 7 mL 53% (w/w) sucrose in 5 mM MES, pH 5.5, 0.5 mM EDTA, 1 mM KCl, 0.1% (v/v) ethanol) (Nuttley *et al.*, 1990). The gradient was subjected to centrifugation for 70 min at 100,000 x *g* (acceleration 2, deceleration 9) in a

Beckman VTi50 rotor, and using a Beckman XL-70 ultracentrifuge. Eighteen 2 mL fractions were collected on ice from the bottom of the tube.

2.10.4.3 Flotation gradient analysis

Peroxisomes were analysed with a two-step sucrose flotation gradient (modified from Heyman *et al.*, 1994). Peak peroxisomal fractions from isopycnic centrifugation (400 µL) were transferred to the bottom of ultracentrifuge tubes and overlaid successively with 2.3 mL of 60% (w/v) sucrose and then 2.3 mL of 35% (w/v) sucrose. Samples were subjected to centrifugation in a Beckman SW50.1 rotor at 200,000 x *g* for 20 h at 4°C. 18 fractions of approximately 250 µL each were collected from the bottom of the tube.

2.10.4.4 Subfractionation and extraction of peroxisomes

The peak fraction of peroxisomes was lysed by the addition of 10 volumes of ice-cold Ti8 buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM PMSF, 1 µg/mL each of leupeptin, pepstatin and aprotinin; Goodman *et al.*, 1990) and incubation on ice for 15 min with occasional agitation. Membranes and associated molecules were pelleted by centrifugation at 200,000 x *g* in a Beckman TLA100.2 rotor for 1 h at 4°C. Proteins from a portion of the resulting supernatant (S_{Ti8}, containing peroxisomal matrix proteins) were precipitated with TCA (Section 2.8.4). The untreated portion of the S_{Ti8} was assayed for protein (Section 2.8.1) and marker enzymes. The peroxisomal membrane pellet (P_{Ti8}) was brought to a final protein concentration of 0.5 mg/mL in ice-cold Ti8 buffer and was treated with either 0.1 M sodium carbonate (Fujiki *et al.*, 1982) or 1% sodium deoxycholate, 1%

(v/v) Triton X-100, 1 M NaCl or 1 M urea (final concentrations). Samples were incubated on ice for 45 min with occasional agitation and then subjected to centrifugation at 200,000 x *g* for 1 h at 4°C in a Beckman TLA100.2 rotor. The resulting pellets were resuspended in Ti8 buffer. Equal amounts of all supernatant and pellet fractions were analysed by SDS-PAGE followed by staining with Coomassie Blue or immunoblotting.

2.10.4.5 Protease protection

A 20KgP, prepared as described in Section 2.10.4.1, but ommitting protease inhibitors, was resuspended in 5 mM MES, pH5.5, 1 M sorbitol. Aliquots (240 µg of protein) were incubated with 0, 10, 20 or 50 µg of trypsin in the absence or presence of 0.5% (v/v) Triton X-100 for 40 min on ice. Reactions were terminated by precipitation of proteins with TCA (Section 2.8.4). Equivalent fractions of each reaction were analysed by SDS-PAGE and immunoblotting.

2.10.4.6 Immunoprecipitation

Anti-SKL and anti-*Yl*Pex5p (Section 2.12) antibodies raised in rabbits were covalently coupled to protein A-Sepharose by the method of Voos *et al.* (1994) and used for immunoprecipitation according to a protocol adapted from the same reference. 250 µL of the S_{Ti8} (Section 2.10.4.4) was diluted 1:1 with 90 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1% (v/v) Triton X-100, 1 mM PMSF, 1 µg/mL each of leupeptin, pepstatin and aprotinin. Any proteins in the diluted S_{Ti8} that might bind protein A-Sepharose nonspecifically were removed by incubation with 50 µL of protein A-Sepharose beads (equilibrated with 10 mM Tris-HCl,

pH 7.5) for 20 min at 4°C. The cleared solution was then incubated with the protein A-Sepharose-linked anti-SKL or anti-*Yl*Pex5p antibodies for 1 h at 4°C. Beads were washed four times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) Triton X-100, and bound proteins were eluted with 100 mM glycine (pH 2.8). Proteins from the flow-through, wash and elution steps were precipitated with TCA (Section 2.8.4) and analysed by SDS-PAGE and immunoblotting.

2.10.4.7 Enzyme assays

Subcellular fractions were routinely assayed for the activities of catalase, 3-hydroxyacyl-CoA dehydrogenase, isocitrate lyase and malate synthase (peroxisomal markers) and cytochrome *c* oxidase (mitochondrial marker). All absorbance measurements were made with a Beckman DU640 spectrophotometer.

2.10.4.7.1 Catalase

Catalase activity was determined essentially by the spectrophotometric method of Lück (1963). Cellular fractions were brought to a final concentration of 50 mM potassium phosphate, pH 7.5, 0.015% (w/v) H_2O_2 in a total volume of 1 mL. Catalase activity was monitored by recording the decrease in the absorbance of H_2O_2 at 240 nm.

2.10.4.7.2 3-Hydroxyacyl-CoA dehydrogenase

The 3-hydroxyacyl-CoA dehydrogenase activity of the multifunctional β-oxidation enzyme was determined by an adaptation of the method of Osumi and Hashimoto (1979).

Cellular fractions were brought to 100 mM Tris-HCl, pH 10.2, 100 mM KCl, 0.01% (w/v) Triton X-100, 1 mM NaN_3, 0.1 mM NAD^+, 0.01 mM β-hydroxybutyryl-C oA in a total volume of 1 mL. 3-Hydroxyacyl-CoA dehydrogenase activity was monitored by recording the increase in absorbance at 340 nm resulting from NADH production.

2.10.4.7.3 Isocitrate lyase

Isocitrate lyase activity was determined by the method of Barth and Weber (1987). Cellular fractions were brought to 50 mM Tris-HCl, pH 8.0, 4.2 mM phenylhydrazine hydrochloride, 2 mM cysteine hydrochloride, 5 mM $MgCl_2$, 2 mM sodium isocitrate in a total volume of 1 mL. Isocitrate lyase activity was monitored by recording the increase in absorbance at 324 nm resulting from glyoxylate production.

2.10.4.7.4 Malate synthase

Malate synthase activity was determined by the method of Dixon and Kornberg (1962). Cellular fractions were brought to a final concentration of 100 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM acetyl-CoA, 1 mM sodium glyoxylate in a total volume of 1 mL. Malate synthase activity was monitored by the decrease in absorbance of acetyl-CoA at 232 nm.

2.10.4.7.5 Cytochrome c oxidase

Cytochrome *c* oxidase activity was determined by the method of Douma *et al* (1985). Cytochrome *c* was reduced by the addition of a spatula tip of sodium dithionite to 80 mg

cytochrome *c*/mL in 50 mM potassium phosphate, pH 7.0, and incubation at room temperature for 15 min. Reduction was judged to be complete when the increase in absorbance at 550 nm reached a plateau. The reducing agent was removed by Sephadex G50 chromatography. Cellular fractions were brought to a final volume of 1 mL in 50 mM potassium phosphate, pH 7.0, containing 40 µM reduced cytochrome *c*. Cytochrome *c* oxidase activity was monitored by recording the decrease in absorbance by reduced cytochrome *c* at 550 nm.

2.11 Genetic analysis of *Y. lipolytica*

2.11.1 Chemical mutagenesis

Y. lipolytica E122 cells were mutagenised by the method of Gleeson and Sudbery (1988). Cells were grown overnight in 20 mL of YEPD, harvested by centrifugation for 5 min at 2,000 x *g* and washed twice with 20 mL each of 0.1 M citrate buffer, pH 5.5. Cells were then resuspended in a small volume of citrate buffer, and an aliquot of the suspension was removed (untreated cells). The remainder of the cells were brought to 20 mL with citrate buffer, following which the mutagen 1-methyl-3-nitro-1-nitrosoguanidine (NTG) was added to 25 µg/mL. The tube was shaken gently and incubated at room temperature for 33 min without further mixing. Various dilutions of the NTG-treated cells were spread on YEPD plates, as were equivalent dilutions of untreated cells, and the plates were incubated at 30°C. The remainder of the cells were washed five times with 5 mL each of sterile water and then resuspended in 5 mL of YEPD. Glycerol was added to aliquots of the cell suspension to a final concentration of 28% (v/v). These were mixed, frozen by liquid

nitrogen and stored at -70°C. NTG treatment resulted in the killing of approximately 70% of the cells, as assessed by comparing the number of colonies arising from treated versus untreated cells after 2 d of growth. A 0.5 mL aliquot of frozen cells was thawed and used to innoculate 5 mL of YEPD. After growth at 30°C for 3.5 h at 180 rpm, an aliquot was diluted 1:10 into fresh YEPD, and 150 µL of the diluted cell suspension was spread on each of 40 YEPD plates. YEPD plates were incubated for 36 h at 30°C. Approximately 800 colonies appeared on each plate. Colonies were replica plated onto YNO and YNA plates containing leucine, uracil and lysine, and incubated at 30°C. Colonies were screened for growth on YNO and YNA after 2-3 d of growth. Colonies showing the desired phenotype (no or poor growth on oleate, robust growth on acetate) were designated ole$^-$ and picked from the master YEPD plates for further analyses.

2.11.2 Screening of the Y. lipolytica genomic DNA library

Strains with the ole$^-$ phenotype were used to screen a *Y. lipolytica* genomic library (Brade, 1992; Nuttley *et al.*, 1993) constructed in pINA445 (Section 2.1.6) by functional complementation. *pex5-1* cells were transformed by electroporation (Section 2.3.2.2), spread onto YNA plates containing uracil and lysine and incubated at 30°C for 2 d. Resulting colonies were replica plated onto YNO plates containing uracil and lysine, and incubation was continued for 2-3 d. Colonies that grew on YNO were rescued to fresh YNA plates. Plasmid DNA was isolated from complemented strains (Section 2.4.2.1) and introduced by electroporation into *E. coli* (Section 2.3.2.1). Plasmids purified from bacteria were transformed into *pex5-1* cells to verify that complementation was linked to the introduction

of plasmid. Complementing plasmids were further analysed by restriction endonuclease mapping, followed by subcloning of fragments into pINA445 and assaying for the ability to restore growth of *pex5-1* on YNO to establish a minimal complementing genomic DNA fragment. The minimal complementing fragment was sequenced (Section 2.6.1).

2.11.3 Integrative disruption of the PEX5 gene

Plasmid p425PEX5 is based on pRS425 (Christianson *et al.*, 1992) and has aa approximately 4.2-kbp insert in the *Hin*dIII site including the entire genomic copy of the *PEX5* gene, with the orientation of the *PEX5* open reading frame (ORF) the same as that of the *LacZ* gene. p425PEX5 was digested with *Sph*I, and ligated to an approximately 2.3 kbp fragment containing the *Y. lipolytica LEU2* gene. In this way, 1654 bp of the coding region and 74 bp of the 3' untranslated region were replaced with the *LEU2* gene. This construct was digested with *Hin*dIII, liberating the *LEU2* gene flanked by approximately 1.3 kbp and approximately 1.4 kbp of the 5' and 3' regions of the *PEX5* gene, respectively. This fragment was used to transform *Y. lipolytica* strains *E122* and *22301-3* to leucine prototrophy. Leu$^-$ strains that were unable to grow on oleic acid were further characterised by Southern blotting (Section 2.6.2).

2.11.4 Mating and complementation analysis

Y. lipolytica strains were mated according to the method of Gaillardin *et al.* (1973). Haploid strains freshly grown on YEPD plates were streaked onto individual PSM plates and incubated at 30°C overnight. A strain of one mating type was streaked as a patch on a YM

plate; a strain of the opposite mating type was streaked at right angles to and on top of the first streak. YM plates were incubated for 4 d at room temperature. Cells from the YM plates were streaked to single colonies twice on YND plates supplemented with the auxotrophic requirements of the desired diploid strain. Diploid strains were recovered to YEPD plates and assayed for their inability to grow on oleate-containing medium.

2.11.5 Sequencing the pex5-1 allele

Genomic DNA was isolated from wild-type and *pex5-1* strains (Section 2.4.2.2). 1 μg of genomic DNA was used as the template for PCR (Section 2.5.9) with the primers 32RI5' and 32RI3' (Table 2-1). Two independent PCR reactions were carried out for each template. PCR products were made blunt with the Klenow fragment of DNA polymerase I, purified by gel electrophoresis and cloned into the *Sma*I site of pGEM7Zf(+). Plasmid inserts were sequenced with primers specific for the *PEX5* gene.

2.11.6 Creation of the pex5-G455D strain

A glycine to aspartic acid substitution at codon 455 and a diagnostic *Xba*I site were introduced into the *PEX5* gene. Plasmid p425PEX5 (Section 2.11.3) was the template for site-directed mutagenesis (Section 2.7) using oligonucleotide G455D (Table 2-1). Mutagenesis products were analyzed by *Xba*I digestion and DNA sequencing. Plasmid p425G455D contained the desired mutation. Plasmid p32ΔX is a construct based in pBluescriptSKII(-) (Stratagene) with the following features: 1) the *Xho*I site of the vector was destroyed by digestion with *Xho*I, followed by blunting of the ends with the Klenow fragment

of DNA polymerase I and religation; 2) the *Eco*RI site contains an *Eco*RI fragment containing the wild-type *PEX5* gene amplified by PCR with primers 32RI5' and 32RI3'. A 352-bp *Nde*I-*Xho*I fragment of plasmid p425G455D containing the engineered mutation was used to replace the equivalent wild-type sequence of plasmid p32ΔX to generate plasmid pGDΔX. The *Y. lipolytica URA3* gene was liberated from pINA443 (Section 2.1.6) with *Sal*I, made blunt with the Klenow fragment of DNA polymerase I and ligated into the blunt-ended *Cla*I site of pGDΔX to yield the integrative plasmid pGDU3.

Y. lipolytica E122 cells were transformed by electroporation (Section 2.3.2.2) with *Bgl*II-digested pGDU3 and plated onto YNA containing leucine and lysine. Ura$^-$ colonies were screened by restriction endonuclease digestion and Southern blot analysis (Section 2.6.2) of their genomic DNA to test for site-specific integration of the plasmid and maintenance of the desired mutation. Strain *E:GDU3*, which contains one wild-type and one mutant copy of the *PEX5* gene flanking the *URA3* gene, was grown in YEPD for approximately 26 h to relax uracil selection. Cells were washed in sterile water and plated onto YNA supplemented with leucine, lysine and uracil and containing 0.075% 5-fluoroorotic acid (5-FOA) to select against cells with a functional *URA3* gene. 5-FOA-resistant colonies were screened as described above to find a strain, *pex5-G455D*, that had lost the *URA3* marker by homologous recombination between the *PEX5* genes and that had retained the mutant allele.

2.12 Generation of anti-*Yl*Pex5p serum

2.12.1 Chimeric protein production

A recombinant fusion protein was generated and purified using the Protein Fusion and Purification System (NEB). A 1734-bp *Sph*I fragment of the *PEX5* gene, which contains both coding region as well as 3' untranslated region and codes for amino acid residues 47-598 of *Yl*Pex5p, was made blunt with T4 DNA polymerase (Section 2.5.4.2) and ligated into the *Xmn*I site of pMAL-c2 (NEB) in-frame and downstream of the ORF encoding *E. coli* maltose binding protein (MBP). The resulting plasmid, pMBP5, encodes the chimeric protein MBP-PEX5.

Bacterial cells harbouring pMBP5 were grown overnight in 10 mL of TYP-ampicillin. The overnight culture was used to inoculate 500 mL of fresh medium, and incubation at 37°C was continued with vigorous aeration. When the culture had reached an OD_{600} of approximately 0.5, IPTG was added to 1 mM and incubation was continued for 2.5 h. Cells were then harvested by centrifugation at 4,000 x *g* for 20 min at 4°C. The cell pellet was resuspended in ice-cold column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF; 10 mL per g of cells). The cell suspension was subjected to two cycles of freezing at -20°C and thawing in ice-water. Cells were sonicated for 30 sec intervals for a total of 4 min using a Branson Sonifier 250 (duty 30%, output control 3). Cells were placed on ice for 30 sec between each sonication. Cell debris was pelleted by centrifugation at 9,000 x *g* for 30 min at 4°C, and the supernatant (crude extract) was retained. A column of amylose resin (approximately 5 mL packed resin) was pre-equilibrated with column buffer at 4°C. The crude extract was diluted 1 in 4 with ice-cold column buffer and was applied to the column at a flow rate of approximately 1 mL/min. The flow-through was collected and reapplied to the column. After washing the column with 15 volumes of

column buffer, bound proteins were eluted with column buffer containing 10 mM maltose (flow rate of approximately 0.2 mL/min). 20 fractions of 1 mL each were collected. The protein profile of a sample of each fraction was determined by SDS-PAGE, followed by staining with Coomassie Brilliant Blue (Sections 2.8.2 and 2.8.3.1, respectively). Peak fractions containing the protein of interest were pooled, boiled in SDS-PAGE sample buffer and analysed by SDS-Page and staining with Coomassie Brilliant Blue.

2.12.2 Recovery of proteins from SDS-polyacrylamide gels

Proteins were recovered after SDS-PAGE essentially by the method of Harlow and Lane (1988). The gel was stained in 0.05% Coomassie Brilliant Blue (R-250) in water for 10-15 min and then destained in water. The region of the gel containing the MBP-PEX5 band was excised with a razor blade, weighed and chopped into small pieces (approximately 3 cm x 0.5 cm). Gel fragments were placed in dialysis tubing along with protein elution buffer (200 mM Tris-acetate, pH 7.4, 1% SDS, 10 mM DTT) at 10 mL buffer per g of wet gel. The dialysis tubing was placed across the width of a large DNA gel box (Hoefer Max Submarine agarose gel unit, Model HE99). Protein running buffer (50 mM Tris-acetate, pH 7.4, 0.1% SDS) was added until the gel slices were completely covered. Electroelution was carried out at 100 V for 3 h at 4°C, after which time the buffer in the dialysis bag was collected and stored at 4°C. Elution and running buffers were replaced, and electroelution was continued at 50 V overnight. The second batch of elution buffer was combined with the first and dialysed against 4 L of 50 mM ammonium bicarbonate (four changes over 24 h). At the least the first dialysis was performed at room temperature to prevent the precipitation

of SDS; subsequent dialyses were performed at 4°C. After dialysis, the protein solution was frozen and lyophilized. The dried protein was dissolved in a minimum volume of water, and a portion of this was analysed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue to assess recovery.

2.12.3 Immunization of animals

Approximately 250 μg of purified MBP-PEX5 was brought to 0.02% SDS in a total volume of 200 μL. An equal volume of Freund's complete adjuvant was added and the solution was made homogeneous by a brief sonication. The mixture was administered by injection at 3-4 subcutaneous sites on a young rabbit (approximately 2.5 kg) or guinea pig (approximately 0.25 kg). Booster injections were administered at 6-week intervals after the initial injection. Booster injections were prepared as described for the initial injection, except that 1) only approximately 125 μg of MBP-PEX5 was used and 2)Freund's incomplete adjuvant was employed. Ten days after each booster injection, a sample of blood was collected from each animal. Ten days after the final booster injection the animals were sacrificed, and all blood was collected. In all cases the serum was decanted from the clot, and any remaining red blood cells were removed by centrifugation at 2,000 x *g* for 5 min. The presence of specific antibodies was assessed by immunoblotting (Section 2.8.3.3).

2.12.4 Purification of antibodies

Rabbit anti-*Yl*Pex5p antibodies were affinity purified essentially by the method of Crane *et al.* (1994). MBP-PEX5 was transferred from an SDS-PAGE gel to Immobilon-P

membrane (Millipore) using a semi-dry electrophoretic transfer system (Section 2.8.3.3). Strips containing MBP-PEX5 were blocked, washed and incubated with anti-*Yl*Pex5p antisera as described for immunoblot analysis except that the serum was used at a dilution of 1:50 or 1:100 in TBST. After 7 washes with TBST (5 min each), the membranes were incubated with ice-cold 0.1 M glycine, pH 2.8 (0.25 mL per cm^2 of membrane) for 3 min at 4°C. The glycine solution was removed and immediately mixed with an equal volume of ice-cold 1 M Tris-HCl, pH 7.5. The membranes were then further incubated for 3 min with ice-cold 0.1 M glycine, pH 2.8 (0.25 mL per cm^2 of membrane), which was pooled with the previous glycine elution. Membranes were immediately washed 7 times (5 min each) with TBST at 4°C. The entire procedure was repeated 4 times. The glycine elutions were pooled and dialyzed against TBS (TBST without the addition of Tween-20). Affinity purified anti-*Yl*Pex5p antibodies were subsequently concentrated 10- to 20-fold by by centrifugation through a Biomax-30 filter (Millipore) at 7,200 x *g* for 10 min at 4°C. Purified antibodies were divided into aliquots and stored at -20°C.

2.13 Heterologous expression of *PEX5* genes

The *PEX5* gene of *Y. lipolytica* was expressed in *P. pastoris* and *vice versa*. Gene expression was confirmed by immunoblot analysis of lysates from oleate-induced cells using antibodies raised against the protein of interest.

2.13.1 Expression of PpPEX5 in Y. lipolytica

Plasmid pTC3, created by Ms. Jennifer Smith and described in Brown (2000), is a

pINA443-based vector designed to facilitate heterologous gene expression in *Y. lipolytica* under peroxisome-proliferating conditions. The plasmid contains a cassette consisting of approximately 950 bp and 730 bp of the 5' and 3' untranslated regions, respectively, of the *Y. lipolytica* thiolase gene (Berninger *et al.*, 1993) separated by a unique *Eco*RI site. Plasmid pTC3P8 was constructed by Ms. Jennifer Smith in the following manner: the plasmid pSP72-pas8 was a gift from Dr. Suresh Subramani (University of California, San Diego, La Jolla, California), which contains as its insert the *P. pastoris PEX5* (formerly *PAS8*) gene modified to have an upstream *Bgl*II site and a mammalian consensus translational initiation site (Kozak, 1986). pSP72-pas8 was digested with *Bgl*II and *Eco*RI to liberate the *PpPEX5* gene. The ends of this fragment were made blunt with the Klenow fragment of DNA polymerase I. The reaction was terminated by extraction with phenol/chloroform, and the DNA was recovered by ethanol precipitation (Sections 2.5.5 and 2.5.6). DNA fragments were then ligated to *Eco*RI adaptors (Gibco/BRL), following which the ligase was inactivated by incubating at 70°C for 10 min. The adaptors were phosphorylated using T4 polynucleotide kinase (Section 2.5.4.2), and the fragment of interest was ligated to *Eco*RI-digested pTC3 in the appropriate orientation. The resulting plasmid, pTC3P8, was used to transform the *Y. lipolytica pex5-KO* strain.

2.13.2 Expression of YlPEX5 in P. pastoris

The *E. coli*/*P. pastoris* shuttle vector pSG464 (Gould *et al.*, 1992) has no elements specifically designed to facilitate gene expression under peroxisome proliferating conditions, so *YlPEX5* gene expression in *P. pastoris* was achieved by using its own endogenous control

elements. An approximately 3.5 kbp piece of *Y. lipolytica* genomic DNA, including the entire *PEX5* ORF plus approximately 1 kbp and 0.7 kbp of its 5' and 3' untranslated regions, respectively, were isolated as a *Hin*dIII-*Eco*RI fragment and ligated into *Hin*dIII-*Eco*RI-digested pSG464. The resulting plasmid, p464EH, was used to transform the *P. pastoris* strain *PPY27* by electroporation (Section 2.3.2.3).

2.14 Plasmids encoding *Yl*Pex5p variants

2.14.1 Plasmids for in vitro transcription/translation

For *in vitro* synthesis of Pex5p and variants, the pKK plasmid series was prepared in pBluescriptSKII(-). Single-stranded DNA from p425PEX5 (Section 2.11.6) was the template for site-directed mutagenesis (Section 2.7) using oligonucleotide KK (Table 2-1) to create a consensus site for mammalian translational initiation (Kozak, 1986), as well as a *Kpn*I site upstream of the initiation codon of *PEX5*. The resulting plasmid (p425KKII) was digested with *Kpn*I and *Cla*I, and the insert was ligated into *Kpn*I-*Cla*I-digested pBluescriptSKII(-) to make plasmid pBSKK. A 1735-bp *Sph*I fragment from plasmid p425PEX5 or plasmid pGDΔX (consisting of nucleotides 139-1873 of the wild-type *PEX5* or *pex5-G455D* genes, respectively) was exchanged against the equivalent region from pBSKK to generate plasmids pKKWT and pKKGD.

Genes encoding truncated variants of *Yl*Pex5p were created by PCR (Section 2.5.9) using the genomic clone of *PEX5* as the template. To create N-terminal truncation mutants, oligonucleotide KKBT, KKCT or KKET was used as the upstream primer (creating a *Kpn*I site upstream of consensus mammalian translation initiation sites as described for

oligonucleotide KK), while KKREV was used as the downstream primer. PCR products were treated with the Klenow fragment of DNA polymerase I, gel purified and ligated into the *Eco*RV site of pGEM5Zf(+) (Promega) to make pG5BT, pG5CT and pG5ET respectively. A 268-bp *Kpn*I-*Eco*RV fragment of pG5BT, a 57-bp *Kpn*I-*Sal*I fragment of pG5CT and a 281-bp *Kpn*I-*Xho*I fragment of pG5ET were exchanged against the equivalent regions of pKKWT to generate plasmids pKKBT, pKKCT and pKKET, respectively. To make C-terminal truncation mutants, oligonucleotide 32RI5' was used as the upstream primer and oligonucleotides BIAT, BIDT or BIFT was used as the downstream primer (creating termination codons followed by a *Sna*BI site). PCR products were digested with *Apa*I and *Sna*BI, and the unique fragment from each reaction was ligated into *Apa*I-*Sna*BI-digested pRcCMV (Invitrogen) to give pRcA, pRcD and pRcF, respectively. A 189-bp *Eco*RV-*Sna*BI fragment of pRcA was ligated into pKKWT digested completely with *Stu*I and partially with *Eco*RV to give pKKAT. A 66-bp *Nde*I-*Sna*BI fragment of pRcD was ligated into *Nde*I-*Stu*I-digested pKKWT to give pKKDT. A 128-bp *Xho*I-*Sna*BI fragment of pRcF was ligated into *Xho*I-*Stu*I-digested pKKWT to give pKKFT. Plasmid pKKBF was created by exchanging the *Kpn*I-*Xho*I fragment of pKKFT for the equivalent region of pKKBT. Plasmid pKKCF was created by exchanging the *Kpn*I-*Nde*I fragment of pKKFT with the equivalent region of pKKCT. pKKAT, pKKDT, pKKFT, pKKBF and pKKCF all lack 156 bp of the 3' untranslated region, from the base after the termination codon to the middle of the *Stu*I site.

2.14.2 Plasmids for in vivo expression

The endogenous *PEX5* promoter was used to drive the *in vivo* expression of genes

encoding truncated Pex5p variants. The *Hin*dIII fragment of p425KKII (Section 2.14.1) was ligated into the *Hin*dIII site of pBluescriptSKII(-) such that the direction of transcription of the *PEX5* gene was opposite to that of the *LacZ* gene. This created plasmid pBSHK which has approximately 1 kbp of *Y. lipolytica* genomic DNA 5' to the initiation codon of *PEX5* located between *Kpn*I sites. The *Kpn*I fragment of pBSHK was ligated into the *Kpn*I sites (in the correct orientation with respect to the ORFs) of the appropriate pKK plasmid to give plasmids pKPAT, pKPDT, pKPFT, pKPBT, pKPCT, pKPET and pKPBF respectively. Promoter-ORF combinations were liberated from the pKP plasmids with *Hin*dIII and ligated into the *Hin*dIII site of pINA443 to give plasmids p443AT, p443DT, p443FT, p443BT, p443CT, p443ET and p443BF, respectively. Expression of the wild-type gene was from plasmid p443KK, consisting of the *Hin*dIII fragment of plasmid p425KKII in the *Hin*dIII site of pINA443. Plasmids were introduced into the *pex5-KO* strain by electroporation (Section 2.3.2.2).

2.15 *In vitro* PTS1 binding assays

2.15.1 Plasmids encoding glutathione S-transferase-PTS1 variant proteins

Variants of *Schistosoma japonicum* glutathione S-transferase (GST) modified at their C-termini were encoded by a series of plasmids based on pGEXQ, a derivative of pGEX4T1 (Amersham-Pharmacia). pGEXQ was constructed as follows: complementary oligonucleotides EQ1 and EQ2 (Table 2-1) were designed so that when annealed, 5' overhangs would be created that were compatible with overhangs resulting from digestion of DNA with *Bam*HI and *Eco*RI, respectively. Oligonucleotides were phosphorylated with

T4 polynucleotide kinase (Section 2.5.3). They were then brought to a final concentration of 25 pmol/μL each in annealing buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$), heated at 90°C for 4 min and allowed to cool slowly to 16°C (Zhang, 1993). The annealing reaction was diluted 25-fold with water, and 0.5 μL of this solution was used in a ligation reaction with *Bam*HI-*Eco*RI - digested pGEX4T1. The result was plasmid pGEXQ, in which the *Eco*RI site was destroyed, creating a codon for glutamine (CAA) in place of glutamic acid (GAA). Plasmids pGEXQ-SKL, pGEXQ-LKS, pGEXQ-AKI and pGEXQ-SKLS (encoding GST-SKL, GST-LKS, GST-AKI and GST-SKLS, respectively) were made by preparing annealed oligonucleotide pairs (SKL1 and SKL2, LKS1 and LKS2, AKI1 and AKI2, and SKLS1 and SKLS2, respectively) (Table 2-1). Annealed oligonucleotides generate *Sal*I- and *Not*I-compatible 5' overhangs, and were ligated into *Sal*I-*Not*I-digested pGEXQ. The sequences of the C-termini of the GST variants are ...LVPRGSPQFPGR(SKL/LKS/AKI/SKLS), respectively. The pGEXQ variant plasmids were transformed into protease-deficient *E. coli* BLR(DE3) cells.

2.15.2 Purification of GST-PTS1 variant proteins

E. coli BLR(DE3) cells carrying a pGEXQ variant plasmid was grown overnight in 5 mL of 2 x YT-ampicillin (Table 2-3) at 37°C with vigorous aeration. The culture was then used to innoculate 500 mL of fresh medium and growth was continued at 37°C until the culture had reached an OD_{600} of approximately 1.0. IPTG was added to a final concentration of 0.1 mM, and the cells were grown at 30°C for 2 h to allow synthesis of the GST proteins. Cells were harvested by centrifugation at 7,700 x *g* for 10 min at 4°C and then subjected to

two cycles of freezing at -20°C and thawing in ice-water. Cells were disrupted by incubation with 20 mL of B-PER bacterial protein extraction reagent (Pierce) for 10 min at room temperature with gentle shaking. Insoluble protein and debris were removed by centrifugation at 27,000 x *g* for 15 min. GST proteins were isolated by passing the supernatant over glutathione-Sepharose beads (Amersham-Pharmacia) pre-equilibrated in PBS. Bound material was eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. Eluted proteins were dialyzed against 2,000 volumes of P8BB binding buffer (20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EDTA, 2 mM DTT) (McCollum *et al.*, 1993) containing 20% (v/v) glycerol at 4°C for 4 h and stored at -80°C until needed.

2.15.3 Purification of MBP-PEX5 and MBP

pMBP5 (Section 2.12.1) and pMAL-c2 were used to produce the MBP-PEX5 chimera and unfused MBP, respectively, in *E. coli* BLR(DE3) cells. MBP-PEX5 and MBP were purified as described in Section 2.12.1. Proteins eluted from the amylose resin were dialyzed and stored as described for GST-PTS1 variant proteins (Section 2.15.2).

2.15.4 Preparation of an E. coli lysate

A lysate of *E. coli* BLR(DE3) cells was prepared according to Ausubel *et al.* (1999) for use as a complex competitor in binding studies. Cells from an overnight culture of LB were harvested by centrifugation at 6,000 x *g*, washed in ice-cold P8BB buffer and repelleted by centrifugation. Cells were resuspended in P8BB buffer (10 mL per L of culture) and then

sonicated with a Branson Sonifier 250 five times for 1 min each (duty 30%, output control 3), with a 2 min pause on ice between each sonication. The sonicate was subjected to centrifugation at 47,800 x *g* at 4°C for 30 min. The supernatant was retained, to which Triton X-100 and glycerol were added to final concentrations of 1% (v/v) and 10% (v/v), respectively. Aliquots of the lysate were stored at -80°C. Before use, the thawed lysate was subjected to centrifugation at 47,800 x *g* at 4°C for 30 min. The supernatant was assayed for protein concentration (Section 2.8.1) and adjusted to 10 mg/mL with P8BB.

2.15.5 PTS1 binding reactions

2.15.5.1 Binding reactions involving purified MBP-PEX5 and MBP

Glutathione-Sepharose beads, pre-equilibrated in P8BB containing 20% (v/v) glycerol, were mixed with an equal volume of 7.5 μg GST variant protein/μL in P8BB containing 20% (v/v) glycerol at room temperature for 30 min. After extensive washing with P8BB containing 10% (v/v) glycerol, 20 μL of the loaded beads was mixed with 200 μL of blocking buffer (P8BB containing 10% (v/v) glycerol, 0.2% (w/v) NP-40, 10 μg *E. coli* BLR(DE3) cell extract (Section 2.15.4)/μL to block non-specific protein binding sites. 5 μg of MBP-PEX5 or an equimolar amount of MBP was mixed with 200 μL of blocking buffer, incubated on ice for 30 min, and subjected to centrifugation at 16,000 x *g* for 10 min. The supernatant was added to the bead mixture and incubated at room temperature for 90 min with gentle mixing. Beads were collected by centrifugation at 500 x *g* for 5 min and the supernatant was removed. Beads were washed with 400 μL each of: 1) P8BB containing 10% (v/v) glycerol, 0.2 % (w/v) NP-40, 2 μg BSA/μL; 2) P8BB containing 10% (v/v)

glycerol, 0.1% (w/v) NP-40, 2 μg BSA/μL; 3) P8BB containing 10% (v/v) glycerol. Wash buffers were ice-cold, and centrifugations were performed at 4°C. After the final wash, all liquid was removed. The drained beads were resuspended in 20 μL of SDS-PAGE sample buffer (Section 2.8.2) and boiled for 5 min to elute bound proteins. Eluted proteins were analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (Sections 2.8.2 and 2.8.3.1). All blocking and wash buffers contained protease inhibitors at the following concentrations: 1μg/mL of each of leupeptin, aprotinin, and pepstatin A, 0.5 mM benzamidine hydrochloride, 5 mM NaF, and 1 mM PMSF. For certain experiments (see text), *E. coli* cell extract and BSA were omitted from the blocking buffer and wash buffers, respectively.

2.15.5.2 Binding reactions involving *in vitro* translated *Yl*Pex5p variants

*Yl*Pex5p variants were produced by *in vitro* translation (Section 2.9.2). An amount of the reticulocyte lysate representing 50,000 precipitated cpm (Section 2.9.3) of wild type Pex5p , or an amount of lysate representing the precipitated cpm of an equimolar amount of a Pex5p variant (unless otherwise specified), was brought to a final volume of 2 μL (unless otherwise specified) with unprogrammed reticulocyte lysate diluted 1:1 with water. The diluted mixture was processed for binding as described in Section 2.15.5.1 for MBP proteins, except that the blocking buffer contained 10 μg BSA/μL instead of *E. coli* lysate. Proteins released from the beads by boiling in SDS-PAGE sample buffer were analysed by SDS-PAGE and fluorography (Section 2.8.3.2). Dried gels were exposed to preflashed Kodak XAR-5 X-ray film at -80°C.

2.16 Computer-aided analyses of nucleic acid and protein sequences

DNA and protein sequences were analysed and compared to other sequences with either the PC-GENE software package (IntelliGenetics), or using the BLAST algorithms (Altschul *et al.*, 1990; Altschul *et al.*, 1997) via the network service (www.ncbi.nlm.nih.gov) of the National Centre for Biotechnology Information (Bethesda, MD.)

CHAPTER 3

Identification and Characterization of the *Yarrowia lipolytica* Peroxin Pex5p

A version of this chapter has previously been published as "Pay32p of the yeast *Yarrowia lipolytica* is an intraperoxisomal component of the matrix protein translocation machinery" (Rachel K. Szilard, Vladimir I. Titorenko, Marten Veenhuis and Richard A. Rachubinski). Reproduced from ***The Journal of Cell Biology*, 1995, 131: 1453-1469** by copyright permission of the Rockefeller University Press.

3.1 Introductory remarks

A genetic screen was undertaken to identify mutants of the yeast *Yarrowia lipolytica* that fail to assemble functional peroxisomes (*pex* mutants). One strain, *pex5-1*, has abnormally small peroxisomes that are often found in clusters surrounded by membranous material. The functionally complementing gene *PEX5* encodes a protein, *Yl*Pex5p, that is a member of the Pex5 family of peroxins. Pex5 proteins have been shown to function as PTS1 receptors in other species. Biochemical characterization of the mutants *pex5-1* and *pex5-KO* (a *PEX5* gene disruption strain) showed that *Yl*Pex5p is a component of the peroxisomal translocation machinery. Mutations in the *PEX5* gene prevent the translocation into the matrix of most, but not all, proteins successfully targeted to peroxisomes. These proteins, including a 62-kD polypeptide recognized by antibodies to a PTS1 (SKL), seem to be trapped in the peroxisomal membrane at an intermediate stage of translocation in *pex5* mutants. *Yl*Pex5p is intraperoxisomal. In wild-type peroxisomes, *Yl*Pex5p is associated primarily with the inner surface of the peroxisomal membrane, but approximately one-third of *Yl*Pex5p is localized to the peroxisomal matrix. The majority of the *Yl*Pex5p in the matrix is complexed with two anti-SKL-reactive polypeptides; the 62-kD protein described above, and a 64-kD protein. In contrast, in peroxisomes of the *pex5-1* mutant, the Pex5 protein is localized exclusively to the matrix and forms no complex with the 62-kD anti-SKL reactive protein stuck in the membrane. The results of the studies presented suggest that there are at least two distinct translocation machineries involved in the import of proteins into peroxisomes.

3.2 Identification of the *pex5-1* mutant

Yeast cells require functional peroxisomes in order to utilize fatty acids as a carbon source, because peroxisomes are the only site of β-oxidation in these organisms (Kunau *et al.*, 1988). This requirement was used to screen for mutants of peroxisome dysfunction in the yeast *Y. lipolytica*, with the goal of isolating strains affected in peroxisome biogenesis. Wild-type *Y. lipolytica* cells were chemically mutagenized and then assayed for their inability to grow on media containing oleic acid as the sole carbon source. From ~6.4 x 10^4 colonies screened, 29 were identified as displaying the ole$^-$ phenotype, *i.e.* demonstrating no growth or impaired growth on oleic acid medium, but robust growth on acetate medium (Table 3-1). Some of the mutants displayed temperature sensitivity or cold sensitivity in the ole$^-$ phenotype. Among these ole$^-$ strains, six have been extensively characterized by various members of the Rachubinski laboratory: mutants #32 (*pex5-1*; formerly known as *pay32-1*, and the subject of this thesis; Szilard *et al.*, 1995; Szilard and Rachubinski (2000), #41 (*pex1-1*; Titorenko *et al.*, 1997; Titorenko and Rachubinski, 1998b), #42 (*pex2-1*; formerly *pay5-1*; Eitzen *et al.*, 1996; Titorenko *et al.*, 1996), #50 (*pex8-1*; formerly *pex17-1*; Smith *et al.*, 1997), #56 (*pex20-1*; Titorenko *et al.*, 1998) and #63 (*pex16-1*; Eitzen *et al.*, 1997). Mutations in the *PEX6* gene seem to be over-represented in this collection of mutants, which is probably due at least in part to the relatively large size (3078 nt) of the *PEX6* ORF (Nuttley *et al.*, 1994).

The growth of wild-type and *pex5-1* mutant cells on acetate- and oleic acid-containing media are shown in Figure 3-1. The wild-type strain grows well on media containing either acetate or oleic acid as the carbon source (Figure 3-1, *E122* + pINA445, compare upper and lower panels). The *pex5-1* mutant, which grows well on acetate plates, cannot grow on oleic

Table 3-1 Characterization of *Y. lipolytica* strains

Strains were grown on YNO medium for 4 d at the temperature indicated. Some strains were tested for functional complementation by transformation with plasmids harbouring previously characterized *Y. lipolytica PEX* genes, or with a *Y. lipolytica* genomic DNA library (Nuttley *et al.*, 1994). The complementing gene, if known, is indicated. *Symbols:* +++, robust growth; ++, impaired growth; +, poor growth; -, no growth; *nt*, not tested.

Strain	Growth on YNO			Complementing gene	Comments/references
	23°C	30°C	33°C		
E122	+++	+++	+++		wild-type
4	+	+	+	nt	Not *pay4* (*pex6*) strain (Nuttley *et al.*, 1994)
8	-	-	-	*PEX6*[a]	
10	+	+	+	nt	
12	-	-	-	*PEX6*[a]	
26	+++	-	-	nt	temperature sensitive
29	+++	-	-	nt	temperature sensitive
32	-	-	-	*PEX5*	this study
38	+	+	+	*PAY38*	Pay38p has strong sequence similarity to the mitochondrial membrane carrier superfamily[b]
39	+++	+++	++	nt	
41	-	-	-	*PEX1*	Titorenko *et al.*, 1997; GenBank accession number AF208231; mutation suppressed by overexpression of a gene encoding a protein with similarity to a translation elongation factor[b]
42	-	-	-	*PEX2*	Eitzen *et al.*, 1996; Titorenko *et al.*, 1996
47	+	+	+	nt	
48	-	-	-	*PEX6*[a]	
50	-	-	-	*PEX8*	Smith *et al.*, 1997
51	-	-	-	*PEX16*[c]	
56	-	-	-	*PEX20*	Titorenko *et al.*, 1998
63	-	-	-	*PEX16*	Eitzen *et al.*, 1997
67	+++	++	-	nt	temperature sensitive
78	++	++	++	nt	
85	+++	+	-	nt	temperature sensitive

94	++	++	++	nt	
95	+	+	-	nt	
99	-	-	-	*PEX6*[a]	
102	-	-	-	*PEX6*[a]	
120	+	+	+	*PAY120*	Pay120p has no significant sequence similarities to known proteins; does not localize to peroxisomes, possibly localized to mitochondria[d]
121	+++	+	-	nt	temperature sensitive
125	-	+	++	nt	cold sensitive
127	++	++	+	nt	
137	-	-	-	*PEX6*[c]	

[a] The mutant strain was transformed with the original genomic DNA library plasmid pO1 (Nuttley *et al.*, 1994), therefore it is possible that a gene adjacent to the *PEX6* locus may be responsible for the complementation, or that *PEX6* overexpression supresses the mutation.
[b] Dr. Melchior Evers, Dr. Richard Rachubinski, personal communication.
[c] Independent clone isolated by transforming the mutant with the genomic DNA library (Ms. Jennifer Smith, personal communication).
[d] Ms. Jennifer Smith, Dr. Vladimir Titorenko, personal communication.

acid plates (Figure 3-1, *pex5-1* + pINA445, compare upper and lower panels). The ability of the *pex5-1* mutant to utilize acetate demonstrates that the ole$^-$ phenotype is not due to a failure of the mitochondria to assimilate acetyl-CoA (the end result of β-oxidation). These data suggest that the *pex5-1* strain fails to grow on oleate medium because of an impairment in peroxisome function.

A mutant could display the ole$^-$ phenotype because of either the deficiency of a single enzyme activity in the β-oxidation pathway (a *fox* mutant, for fatty acid oxidation), or because of a more general defect in peroxisome assembly (a *pex* mutant). Which of these two possibilities was true for the *pex5-1* mutant was initially investigated by indirect

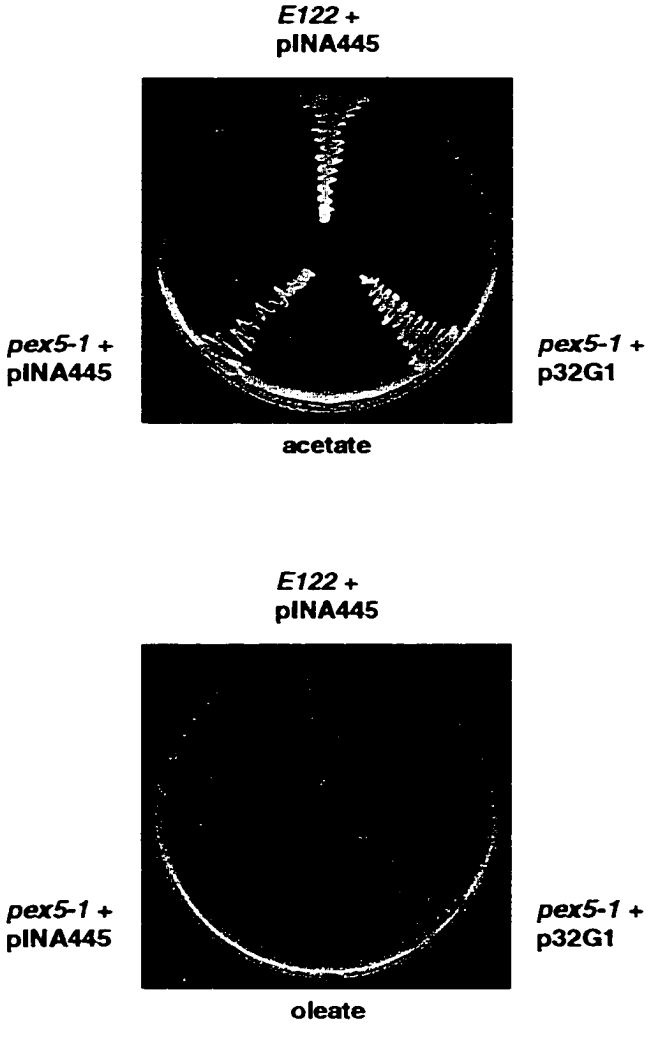


Figure 3-1. The inability of the *pex5-1* mutant to grow on oleate medium is restored by transformation with plasmid p32G1. The wild-type and *pex5-1* mutant strains, each transformed with the plasmid vector pINA445 (*E122* + pINA445 and *pex5-1* + pINA445, respectively), and the *pex5-1* strain transformed with complementing plasmid p32G1 were streaked onto YNA (*upper panel*) or YNO (*lower panel*) and incubated at 30°C for 3 d (YNA) or 5 d (YNO).

immunofluorescence using anti-SKL antiserum. In wild-type cells a punctate pattern of fluorescence was seen, which is characteristic of peroxisomal staining (Figure 3-2, *E122*). In contrast, staining of *pex5-1* cells revealed a diffuse cytoplasmic fluorescence (Figure 3-2, *pex5-1*), suggesting that anti-SKL-reactive proteins are not correctly imported into peroxisomes, and that this ole⁻ strain is indeed a *pex* mutant.

3.3 The *pex5-1* mutant has abnormal peroxisome morphology

Analysis of the wild-type strain and the *pex5-1* mutant by electron microscopy and immunocytochemistry was performed by Dr. Marten Veenhuis (University of Groningen, The Netherlands). Ultrastructural analysis of wild-type cells grown on oleic acid medium (YPBO) shows large, round peroxisomes, each circumscribed by a single unit membrane, and well separated from one another (Figure 3-3 A). In contrast, peroxisomes in *pex5-1* mutant cells are generally smaller than those in wild-type cells, and are often found in clusters surrounded by large membranous structures of unknown origin and composition (Figure 3-3 B). However, small individual peroxisomes are also seen in the mutant strain (Figure 3-3 D). The proliferation of peroxisome-associated membranous structures has also been noted for *pex5* mutants of *H. polymorpha* (van der Klei *et al.*, 1995) and *S. cerevisiae* (van der Leij *et al.*, 1992).

Immunocytochemistry performed on wild-type cells using anti-thiolase (Figure 3-4 E) or anti-SKL (Figure 3-4 F) antibodies demonstrated intense labelling of peroxisomes. Similar analysis of *pex5-1* cells showed that anti-thiolase (Figure 3-4 A) and anti-SKL (Figure 3-4 B) antibodies labelled peroxisomal structures in this strain. The membranous complexes

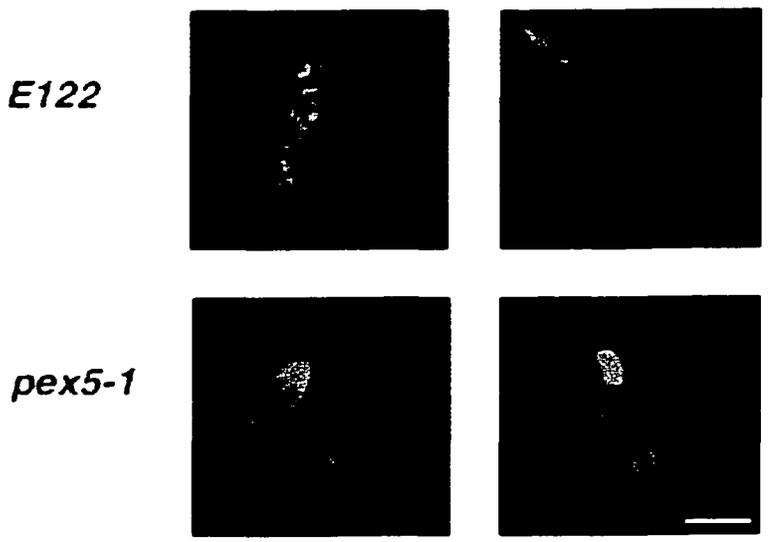


Figure 3-2. Immunofluorescence microscopic analysis of wild-type and *pex5-1* mutant strains. The wild-type (*E122*) and *pex5-1* mutant (*pex5-1*) were precultured in YEPD and then transferred to YPBO for 10 h. Cells were processed for immunofluorescence microscopy with rabbit anti-SKL and rhodamine-conjugated goat anti-rabbit IgG antibodies as described in Section 2.10.1. Images were captured with a digital camera. *Bar*, 5 μm.

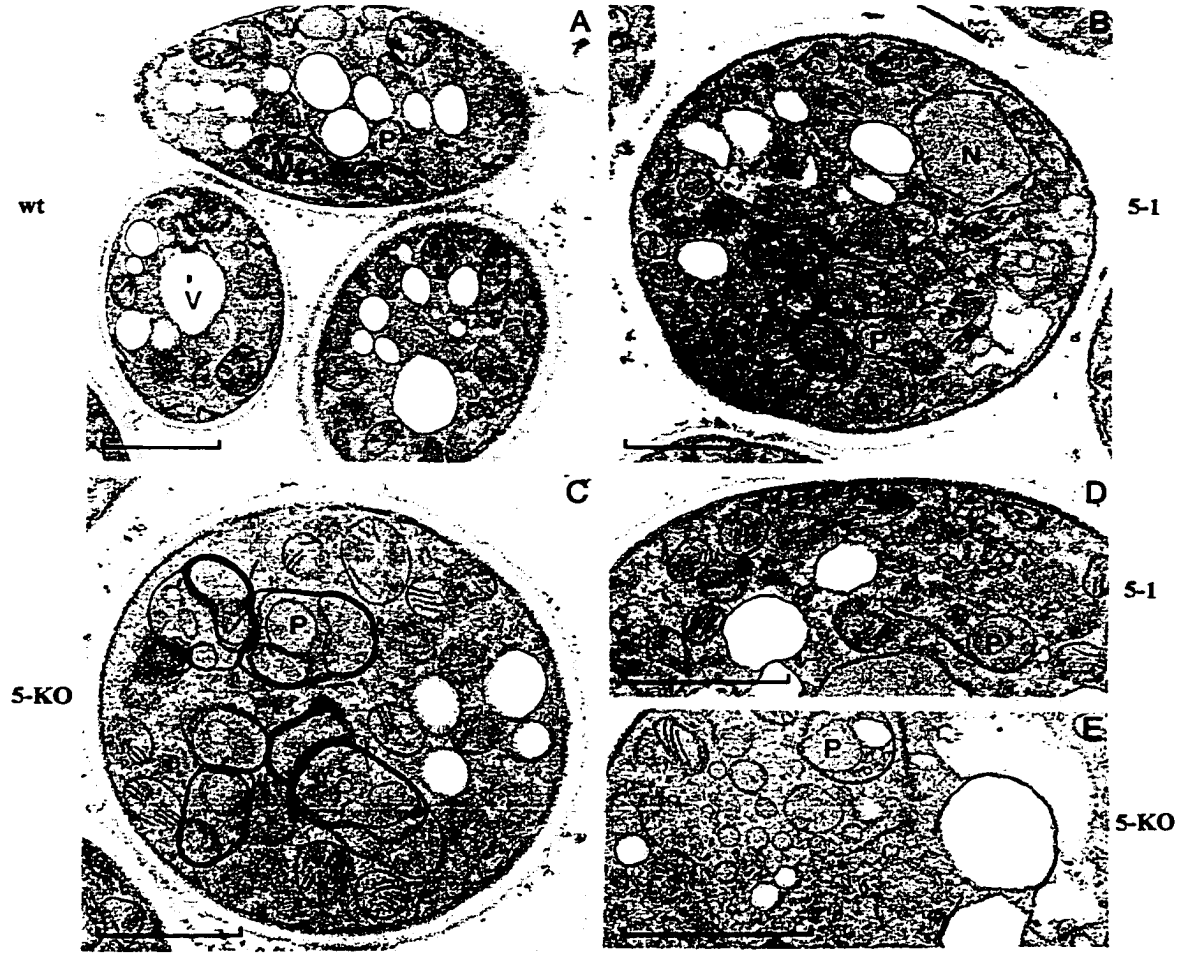


Figure 3-3. Ultrastructure of the wild-type and *pex5* mutant strains. The wild-type (*A*), *pex5-1* (*B and D*), and *pex5-KO* (*C and E*) strains were grown to saturation on YEPD, transferred to YPBO and grown for an additional 8 h. Cells were fixed in $KMnO_4$ and processed for electron microscopy. *P*, peroxisome; *M*, mitochondrion; *N*, nucleus; *V*, vacuole. *Bar*, 1 μm.

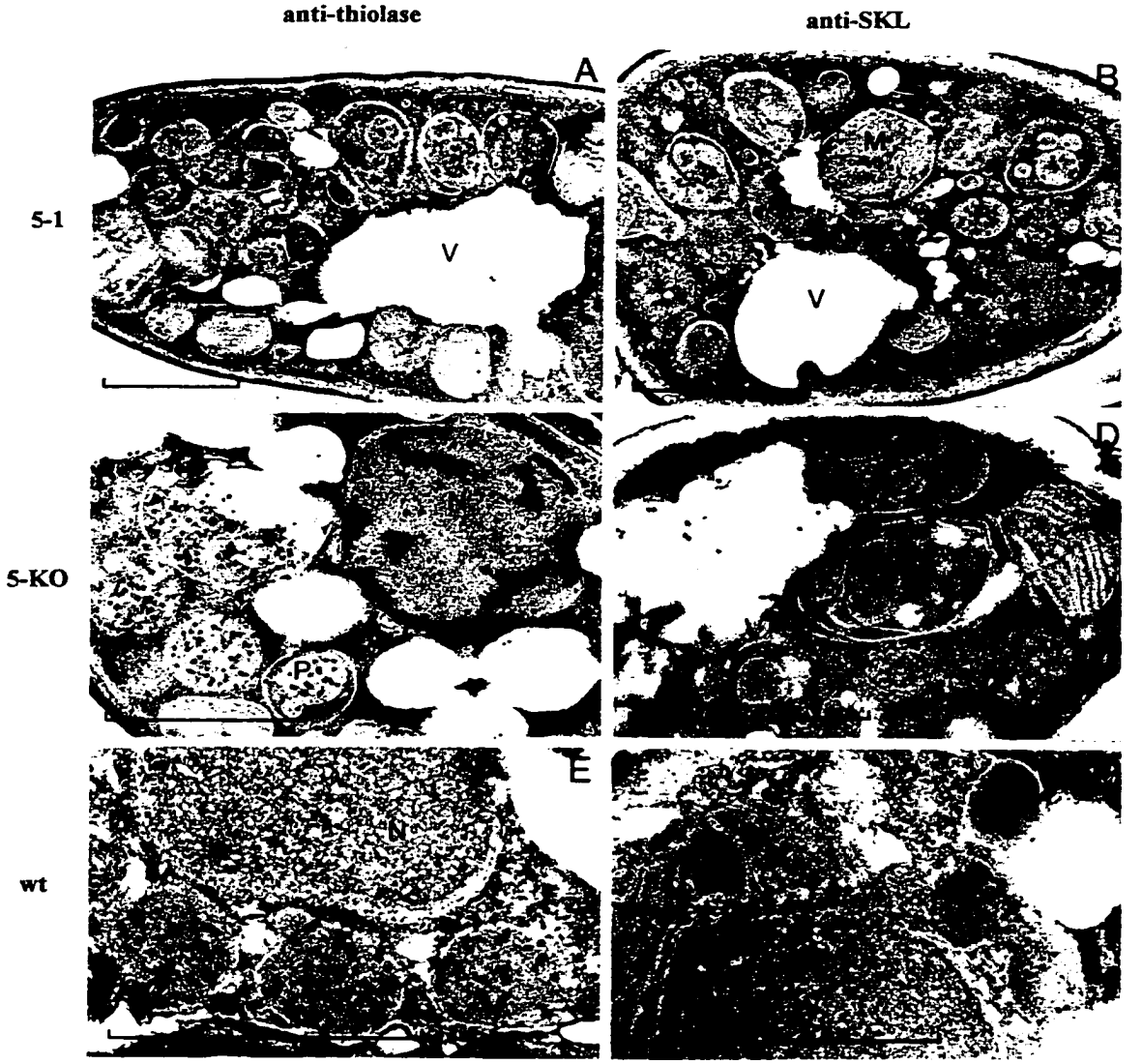


Figure 3-4. Immunocytochemical analysis of wild-type and *pex5* mutant strains. The wild-type (*E and F*), *pex5-1* (*A and B*) and *pex5-KO* (*C and D*) strains were grown as described in the legend to Figure 3-3. The cells were fixed with glutaraldehyde/formaldehyde and processed for immunoelectron microscopy with anti-thiolase (*A, C and E*) or anti-SKL (*B, D and F*) antibodies. *Symbols* are as for Figure 3-3. *Arrows* point to membranous complexes.

surrounding many peroxisomes in *pex5-1* cells were also labelled with anti-thiolase and anti-SKL sera (arrows in Figure 3-4 A and B), thus suggesting their peroxisomal nature. The results of the immunolabelling experiments suggest that thiolase and one or more anti-SKL-reactive proteins are targeted to peroxisomal structures in the *pex5-1* mutant.

3.4 Cloning and sequence analysis of the *PEX5* gene

The *PEX5* gene was isolated from a *Y. lipolytica* genomic DNA library in the autonomously replicating *E. coli* shuttle vector pINA445 (Brade, 1992; Nuttley *et al.*, 1993) by functional complementation of the ole$^-$ phenotype of *pex5-1* cells. Of the ~5 x 10^3 transformants screened, one had regained the ability to grow on oleic acid (Figure 3-1 lower panel, compare *pex5-1*− p32G1 with *pex5-1* + pINA445). This transformant carried the plasmid p32G1, which contains an ~5.4 kbp insert of *Y. lipolytica* genomic DNA. An ~4.1 kbp region and an overlapping ~3.5 kbp region were sufficient to functionally complement the *pex5-1* growth defect on oleic acid (Figure 3-5). Sequencing within the 3.5 kbp region revealed an ORF of 1794 bp encoding a conceptual protein of 598 amino acid residues (Figure 3-6) with a predicted molecular mass of 66,733 D. This protein was originally designated Pay32p (Szilard *et al.*, 1995), but was later renamed *Yl*Pex5p when peroxin nomenclature was unified (Distel *et al.*, 1996). The sequence of *Yl*Pex5p shows extensive similarity to other members of the Pex5 peroxin family (McCollum *et al.*, 1993; van der Leij *et al.*, 1993; Nuttley *et al.*, 1995; van der Klei *et al.*, 1995; Fransen *et al.*, 1995; Dodt *et al.*, 1995; Wiemer *et al.*, 1995; Baes *et al.*, 1997; Otera *et al.*, 1998; Wimmer *et al.*, 1998; Kragler *et al.*, 1998; Brickner *et al.*, 1998; de Walque *et al.*, 1999), especially in the

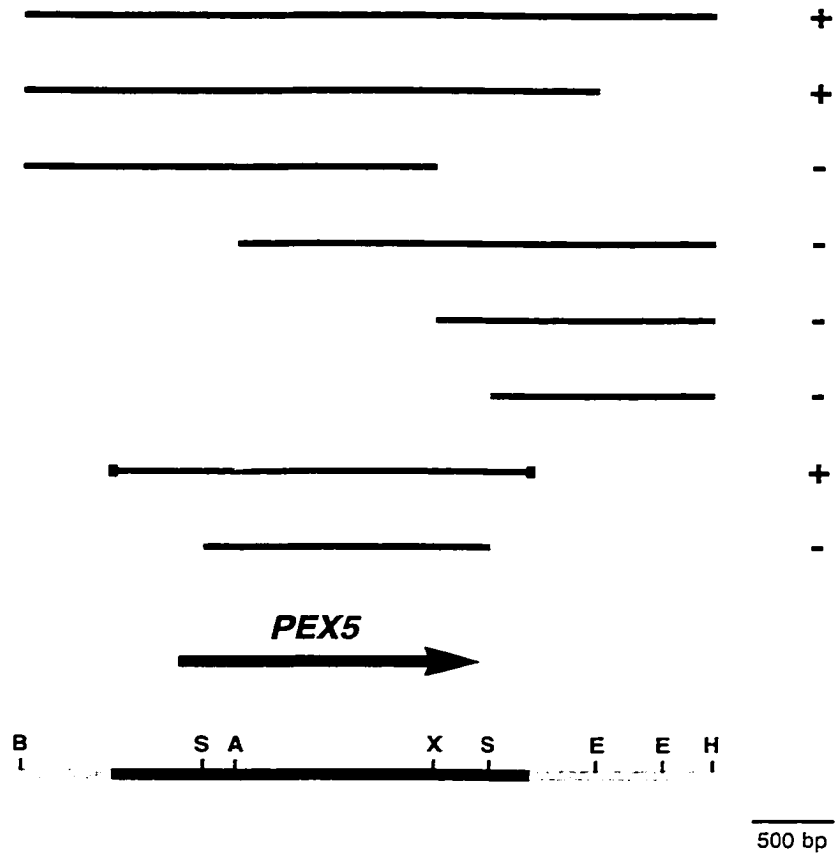


Figure 3-5. **Identification of the *Y. lipolytica PEX5* gene.** The longest line represents the 4.1 kbp-fragment from the original insert of p32G1. A restriction endonuclease map is shown at the bottom of the illustration. The arrow indicates the direction and position of the ORF of the *PEX5* gene. The (+) and (-) symbols denote the ability and inability, respectively, of a fragment to confer growth on oleic acid to *pex5-1*. The line with the boxed ends represents the region amplified by PCR with the oligonucleotides 32RI5' and 32RI3' (Table 2-1). The grey areas on the map indicate regions that were not sequenced. *A*, *Apa*I; *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *S*, *Sph*I; *X*, *Xho*I.

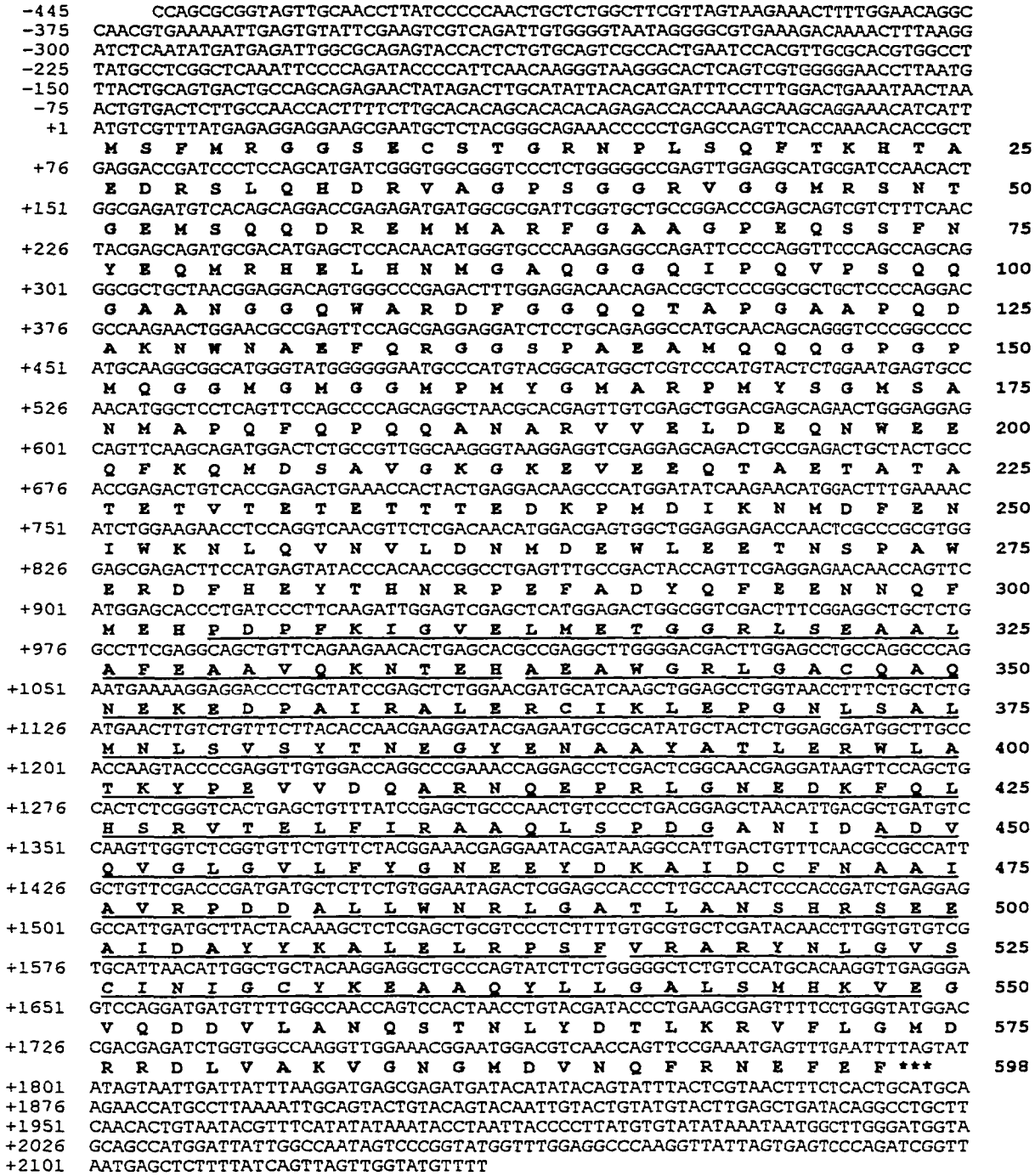


Figure 3-6. Sequence analysis of the *Y. lipolytica PEX5* gene. The nucleotide sequence of the *PEX5* gene and the deduced amino acid residue sequence of *Yl*Pex5p are shown. The TPR motifs are underlined. The numbers at left indicate nucleotide positions. The numbers at right indicate amino acid residue positions. The nucleotide sequence reported here has the GenBank accession number U28155.

characteristic TPR domain in the C-terminal half of the protein. The TPR domain of Pex5 proteins consists of seven repeats of the 34-amino acid residue motif and has been implicated in PTS1 binding (McCollum *et al.*, 1993; Brocard *et al.*, 1994; Terlecky *et al.*, 1995; Fransen *et al.*, 1995; Dodt *et al.*, 1995; Wiemer *et al.*, 1995; Kragler *et al.*, 1998). An alignment of Pex5 protein sequences is provided in Appendix 1.

The N-termini of Pex5 family members contain multiple copies of a pentapeptide motif with the consensus sequence WXXXF/Y, with different numbers of motifs and spacings in individual proteins (Dodt *et al.*, 1995; Kragler *et al.*, 1998; Wimmer *et al.*, 1998; Schliebs *et al.*, 1999; de Walque *et al.*, 1999). These motifs have been suggested to be involved in the interaction of human Pex5p and Pex14p (Schliebs *et al.*, 1999), but this theory has not yet been addressed experimentally. In *Yl*Pex5p, there are four copies of the pentapeptide motif, starting at amino acid residues 108, 129, 198 and 275 (Schliebs *et al.*, 1999).

The sequence of *Yl*Pex5p also shows similarity to the *Y. lipolytica* peroxin Pex20p (Titorenko *et al.*, 1998), which is required for the oligomerization of thiolase and for its targeting to peroxisomes. *Yl*Pex20p has no TPR domain, and the region of similarity is restricted to the N-terminal region of *Yl*Pex5p (Titorenko *et al.*, 1998).

3.5 *Yl*Pex5p synthesis is induced by growth of *Y. lipolytica* on oleic acid

Wild-type cells were precultured in glucose medium (YEPD) and then transferred to oleic acid medium (YPBO) to induce peroxisome formation. Extracts from cells harvested at various times during the induction period were analysed for the levels of *Yl*Pex5p, thiolase, Kar2p (a marker for the endoplasmic reticulum) and glucose-6-phosphate dehydrogenase (a

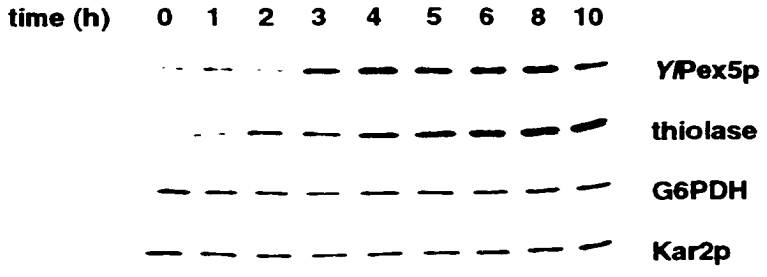


Figure 3-7. *Yl*Pex5p synthesis is induced by growth of *Y. lipolytica* in oleic acid-containing medium. The wild-type strain *E122* was precultured in YEPD to an OD_{600} of 1.3 and then transferred to YPBO at an initial OD_{600} of 0.016. Samples were removed at various times after transfer to YPBO (*numbers at top*) and lysates were prepared by disruption with glass beads. Lysates (1 μg) of cells were analysed by immunoblotting with antibodies to*Yl*Pex5p, thiolase, Kar2p and glucose-6-phosphate dehydrogenase (*G6PDH*).

cytosolic protein) (Figure 3-7). *Yl*Pex5p is present in YEPD-grown cells. Following transfer to YPBO, the level of *Yl*Pex5p rises, as does that of thiolase, a protein encoded by a gene whose expression is known to be oleate-inducible (Eitzen *et al.*, 1995). The level of *Yl*Pex5p peaks by 4 h and remains elevated for the duration of the induction, although declining slightly at the last time point. The changes in the level of *Yl*Pex5p was not due to a non-specific increase in cellular protein synthesis following transfer to YPBO, since the levels of Kar2p and glucose-6-phosphate dehydrogenase do not show similar increases over the course of the induction.

Based on its electrophoretic mobility, the specific protein recognized by the anti-*Yl*Pex5p antibodies runs with an apparent molecular mass of ~71 kD (Figure 3-10 B, lane *E122*), which is slightly larger than the predicted molecular mass of this protein (66,733 D); this is a feature common to several Pex5 family members (van der Klei *et al.*, 1995; Wiemer *et al.*, 1995; Dodt *et al.*, 1995; Baes *et al.*, 1997; Wimmer *et al.*, 1998; Schliebs *et al.*, 1999).

3.6 Integrative disruption of the *PEX5* gene and complementation analysis

The *Y. lipolytica LEU2* gene was used to disrupt the *PEX5* gene (Figure 3-8 A). The disruption construct was used to transform the wild-type strains *E122* and *22301-3* to leucine prototrophy, creating strains *pex5-KO* and *pex5-KOB* in the A and B mating types, respectively. The genomic DNA of Leu$^-$ strains was analysed by Southern blotting to ensure site-specific integration of the *LEU2* gene (Figure 3-8 B). Strains with a disrupted *PEX5* gene could not grow on oleic acid (Figure 3-9, compare *pex5-KO* and *pex5-KOB* to *E122* and

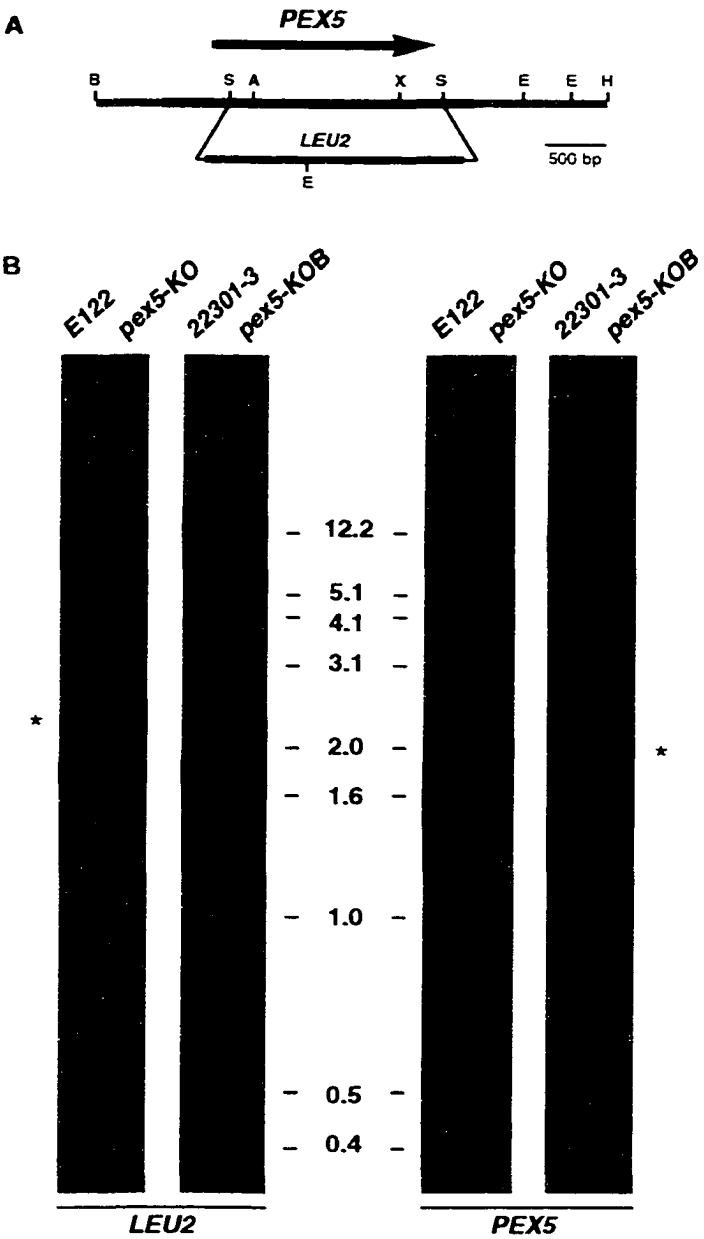


Figure 3-8. Targeted integrative disruption of the *PEX5* gene. (*A*) Gene disruption strategy. The site of integration of the *Y. lipolytica LEU2* gene in the *PEX5* gene is shown. The ORF of the *LEU2* gene is in the same direction as that of the *PEX5* gene. *Symbols* are as in Figure 3-5. (*B*) Southern blot analysis of wild-type (*E122* and *22301-3*) and *pex5* disruption strains (*pex5-KO* and *pex5-KOB*). Genomic DNA was isolated, digested with *Eco*RI and processed for Southern blotting as described in Section 2.6.2.1. The probe for the *LEU2* sequence was the *Sph*I fragment used in the disruption construct. The probe for the *PEX5* sequence was the 4.1-kbp fragment from plasmid p32G1. The *asterisk* indicates the band that is diagnostic for site-specific integration of the *LEU2* gene into the *PEX5* gene. *Numbers* represent the sizes of molecular markers (in kbp).

22301-3, respectively) and had the same abnormal peroxisomal morphology (Figure 3-3 C and E; Figure 3-4 C and D) and protein import defects (see below) as did the original *pex5-1* mutant.

E122, *pex5-1* and *pex5-KO* were each mated with *22301-3* and *pex5-KOB* to yield diploid strains (see Table 3-1) used for complementation analysis (Figure 3-9). The diploid strain *D5WTB*, resulting from the mating of the *pex5-1* mutant and the wild-type strain *22301-3*, could grow on oleic acid, demonstrating the recessive nature of the *pex5-1* mutation. A diploid strain resulting from the mating of *pex5-1* and *pex5-KOB* (*D5KOB*) was unable to grow on oleic acid, demonstrating that the cloned gene was indeed *PEX5*, and not an extragenic suppressor of the original mutation.

Y. lipolytica and *P. pastoris PEX5* genes were tested for their ability to complement when expressed heterologously. The *YlPEX5* gene was expressed in the *P. pastoris pex5-1* mutant (strain *PPY27*; Gould *et al.*, 1992), in which no *Pp*Pex5p is detectable (McCollum *et al.*, 1993); similarly, the *PpPEX5* gene was expressed in the *Y. lipolytica pex5-KO* strain. In neither case did complementation of the oleic acid growth defect of the mutant occur (Figure 3-10 A), although the heterologous protein was detectable in whole cell lysates (Figure 3-10 B). Similar results have been reported for other interspecies complementation experiments involving *PEX5* genes (Dodt *et al.*, 1995; Wiemer *et al.*, 1995; Kragler *et al.*, 1998; Wimmer *et al.*, 1998).

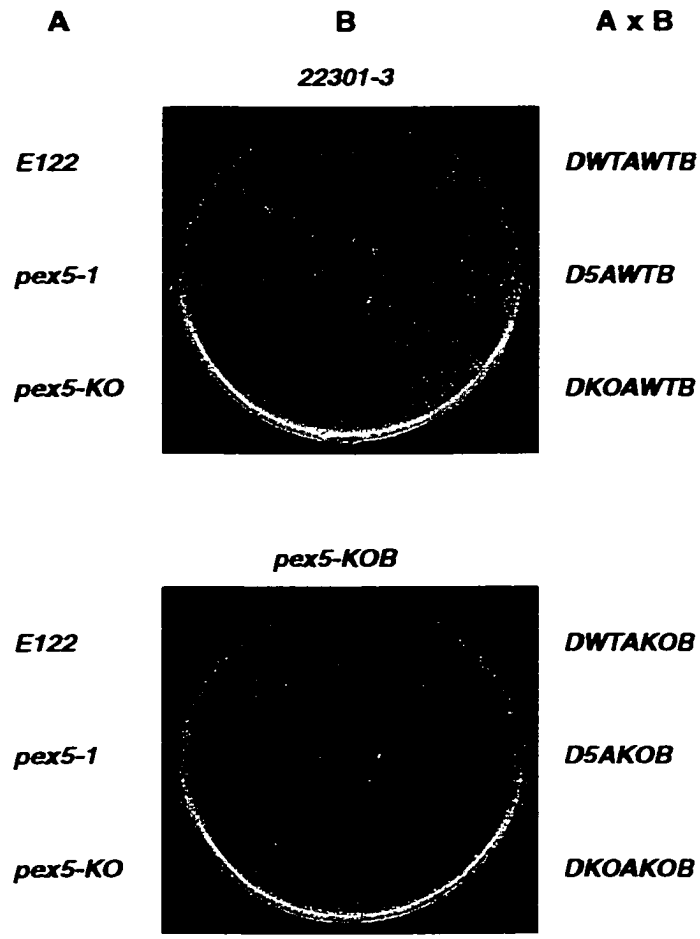


Figure 3-9. Growth of various strains on oleate medium. Haploid (*A*, and *B*) and diploid (*AxB*) strains (refer to Table 2-4 for genotypes) were streaked onto YNO and grown for 5 days at 30°C. Growth on YNO requires at least one copy of the intact *PEX5* gene.

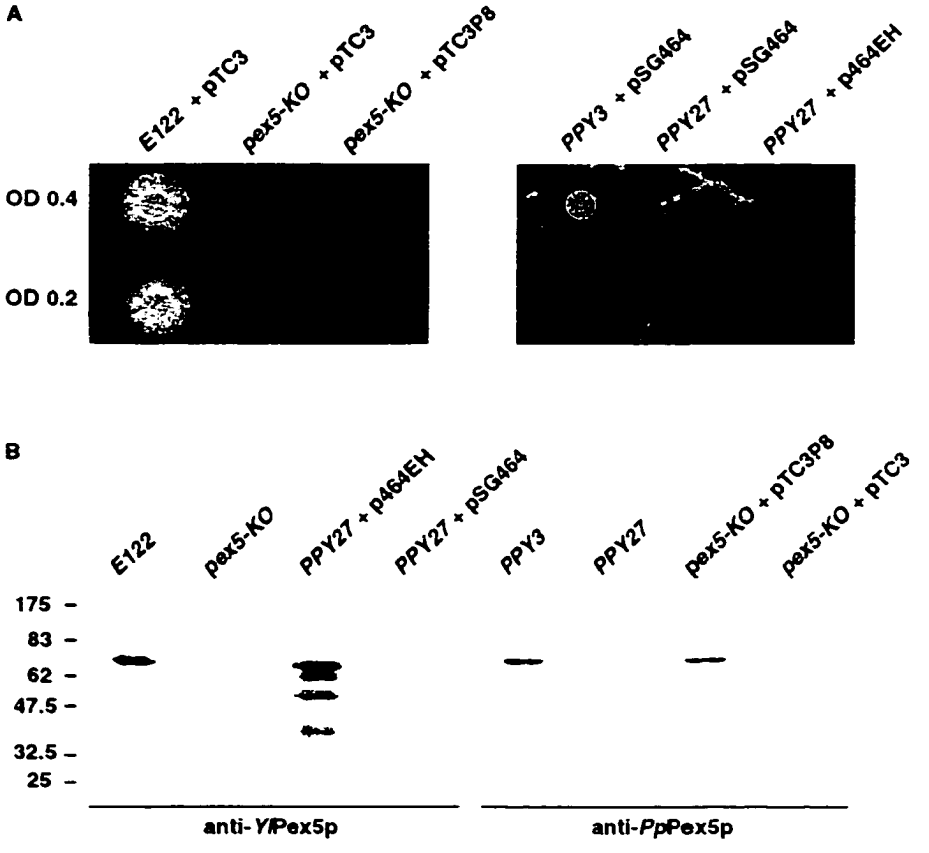


Figure 3-10. Heterologous expression of *PEX5* genes in *Y. lipolytica* and *P. pastoris*. (*A*) Functional complementation analysis. The *Y. lipolytica* wild-type strain *E122* transformed with the plasmid vector pTC3 (*E122* + pTC3) and the *pex5-KO* strain transformed with pTC3 or TC3P8 (*pex5-KO* + pTC3 and *pex5-KO* + pTC3P8) were precultured in 2xCMD. The *P. pastoris* wild-type strain *PPY3* transformed with the plasmid vector pSG464 (*PPY3* + pSG464) and the *P. pastoris pex5-1* strain *PPY27* transformed with pSG464 or p464EH (*PPY27* + pSG464 and *PPY27* + p464EH, respectively) were precultured in YND. The cultures were diluted to an OD_{600} of 0.4 or 0.2, and 3 µL of the cell suspensions were plated on 2xCMO (*Y. lipolytica* strains) or SOLT (*P. pastoris* strains). Growth was for 5 days at 30°C. (*B*) Synthesis of heterologous Pex5 proteins. The untransformed *Y. lipolytica* wild-type (*E122*) and *pex5-KO* (*pex5-KO*) strains and the *pex5-KO* strain transformed with plasmid pTC3 or pTC3P8 (as described above) were precultured in 2xCMD and then transferred to 2xCMO for 6 h. The untransformed *P. pastoris* wild-type (*PPY3*) and *pex5-1* (*PPY27*) strains and the *P. pastoris pex5-1* strain transformed with the plasmid pSG464 or p464EH (as described above) were precultured in YND and transferred to SOLT for 6 h. Lysates of cells (25 µg) made by disruption with glass beads were analysed by immunoblotting with anti-*Yl*Pex5p or anti-*Pp*Pex5p antiserum. *Numbers* on the left indicate the migrations of molecular size standards (in kD).

3.7 Mutations in the *PEX5* gene affect the subcellular localization of peroxisomal proteins

To determine the effects of the *pex5-1* and *pex5-KO* mutations on the import of peroxisomal proteins, wild-type and mutant strains were precultured in glucose-containing medium (YEPD) and then transferred to oleic acid-containing medium (YPBO) for 9 h to induce peroxisome formation. The levels of the peroxisomal matrix proteins analysed were comparable in wild-type, *pex5-1* and *pex5-KO* cells (Figure 3-11, *upper panels* [catalase (CAT), isocitrate lyase (ICL), malate synthase (MLS), and the multifunctional β-oxidation enzyme (3-hydroxyacyl-CoA dehydrogenase activity; HAD)]; Figure 3-14 A, thiolase; Figure 3-15 A, acyl-CoA oxidase; Figure 3-15B, anti-SKL reactive proteins). However, the subcellular distribution of these proteins in the *pex5-1* and *pex5-KO* strains was different than that in the wild-type strain. While the peroxisomal proteins in the wild-type strain were primarily found in the 20,000 x *g* pellet (20KgP, enriched for peroxisomes and mitochondria), these proteins were partially or completely mislocalized to the 20,000 x *g* supernatant (20KgS, enriched for cytosol) in *pex5* mutant cells (Figure 3-11, *middle and lower panels*; Figure 3-14 A; Figure 3-15 A and B). Recent experimentation has revealed that a portion of the thiolase localized to the 20KgS was present in the pellet fraction following a centrifugation at 200,000 x *g* for 1 h. This suggests that the thiolase pelletable at high speed might be present in low density vesicular structures. In contrast, all acyl-CoA oxidase and isocitrate lyase were present exclusively in the supernatant of the 200,000 x *g* centrifugation, suggesting that both of these proteins are free in the cytosol (Dr. Vladimir Titorenko, personal communication).

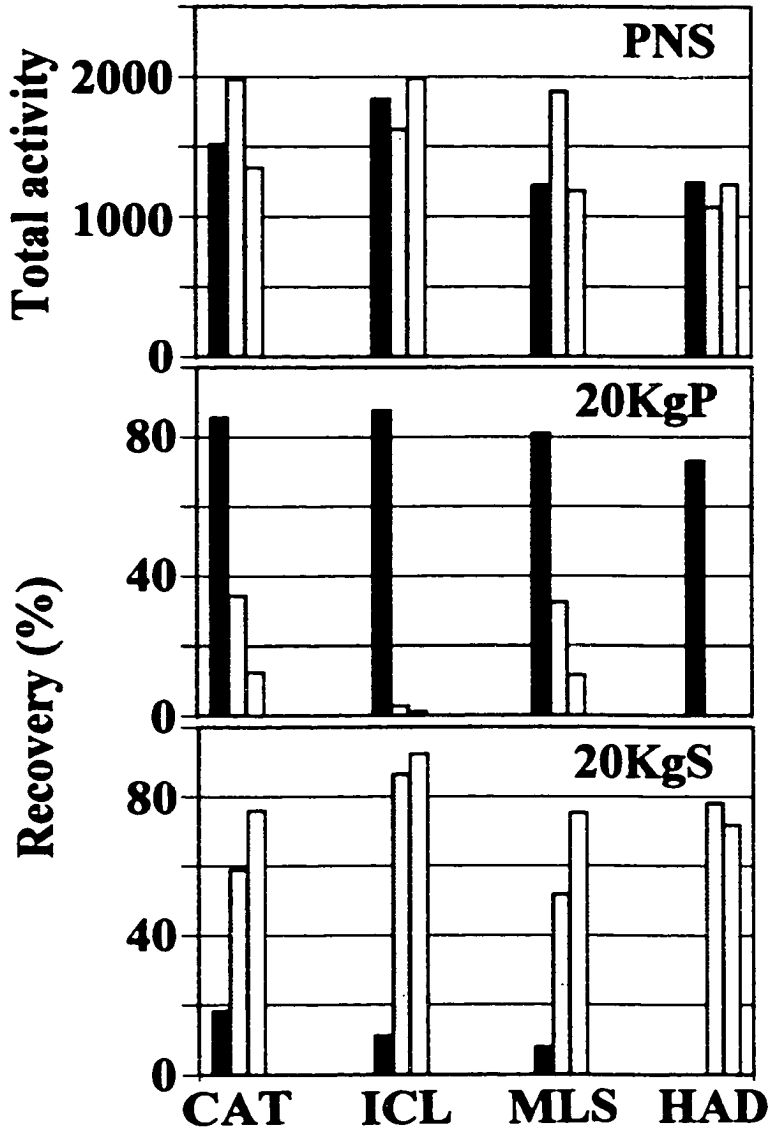


Figure 3-11. Peroxisomal proteins are induced normally by oleic acid but are mislocalized to the cytosol-enriched fraction in *pex5* mutant cells. Wild-type (*solid bar*), *pex5-1* (*stippled bar*), and *pex5-KO* (*open bar*) strains were grown in YEPD for 10 h, transferred to YPBO medium and grown for an additional 9 h. Cells were subjected to subcellular fractionation as described in Section 2.10.4.1. The total enzymatic activities of catalase (*CAT*), isocitrate lyase (*ICL*), malate synthase (*MLS*) and the multifunctional β-oxidation enzyme (3-hydroxyacyl-CoA dehydrogenase activity; *HAD*) in 50 mg of protein of post-nuclear supernatant (*PNS*) are expressed either in U (*CAT*), mU (*ICL*), or mU x 10 (*HAD*) (*top panel*). The percentages of total enzymatic activity recovered in the 20KgP (*middle panel*) and 20KgS (*bottom panel*) are shown. *Contributed by Dr. Vladimir Titorenko.*

The 20KgP fractions were further analysed by sucrose density gradient centrifugation. In the wild-type strain, the peak activities of the peroxisomal marker enzymes isocitrate lyase, malate synthase and catalase were recovered in fraction 4, which had a density of 1.21 g/cm^3 (Figure 3-12 A, *panels wt*). Immunoblotting revealed that the fraction with peak peroxisomal enzyme activity also had the highest concentrations of thiolase (Figure 3-12 B, *panel wt*) and of two anti-SKL-reactive proteins (Figure 3-12 C, *panel wt*): the upper protein that runs with an apparent molecular mass of ~64 kD (p64) is apparently isocitrate lyase (Eitzen, 1997); the lower protein with an apparent molecular mass of ~62 kD (p62) has not been identified. Analysis of gradient fractions from preparations of *pex5-1* and *pex5-KO* cells revealed that fraction 2 (density 1.24 g/cm^3) contained the peak activities of isocitrate lyase, malate synthase and catalase, as well as the highest concentrations of thiolase and anti-SKL reactive proteins (Figure 3-12 A, B and C, respectively, *panels 32-1* and *32-KO*). The reason for the aberrant migration of peroxisomes from *pex5* mutant strains in sucrose gradients is unknown. The recovery of peroxisome-associated protein as a fraction of total protein from the 20KgPs was 26.2%, 12.4% and 3.8% for wild-type, *pex5-1* and *pex5-KO*, respectively (Figure 3-12 A). The lower recovery of peroxisome-associated proteins from *pex5* mutant cells probably reflects the mislocalization of proteins normally found in the peroxisomal matrix to the cytosolic fraction. The mitochondrial marker enzyme cytochrome *c* oxidase peaked in fraction 10 and was essentially absent from fractions 1-6 of the wild-type gradient, and also from fractions 1-4 of the *pex5-1* and *pex5-KO* gradients (Figure 3-12 A). Therefore peroxisomes isolated from all strains were free of contamination from mitochondria.

Immunoblotting with antiserum raised to *Yl*Pex5p revealed that the wild type and

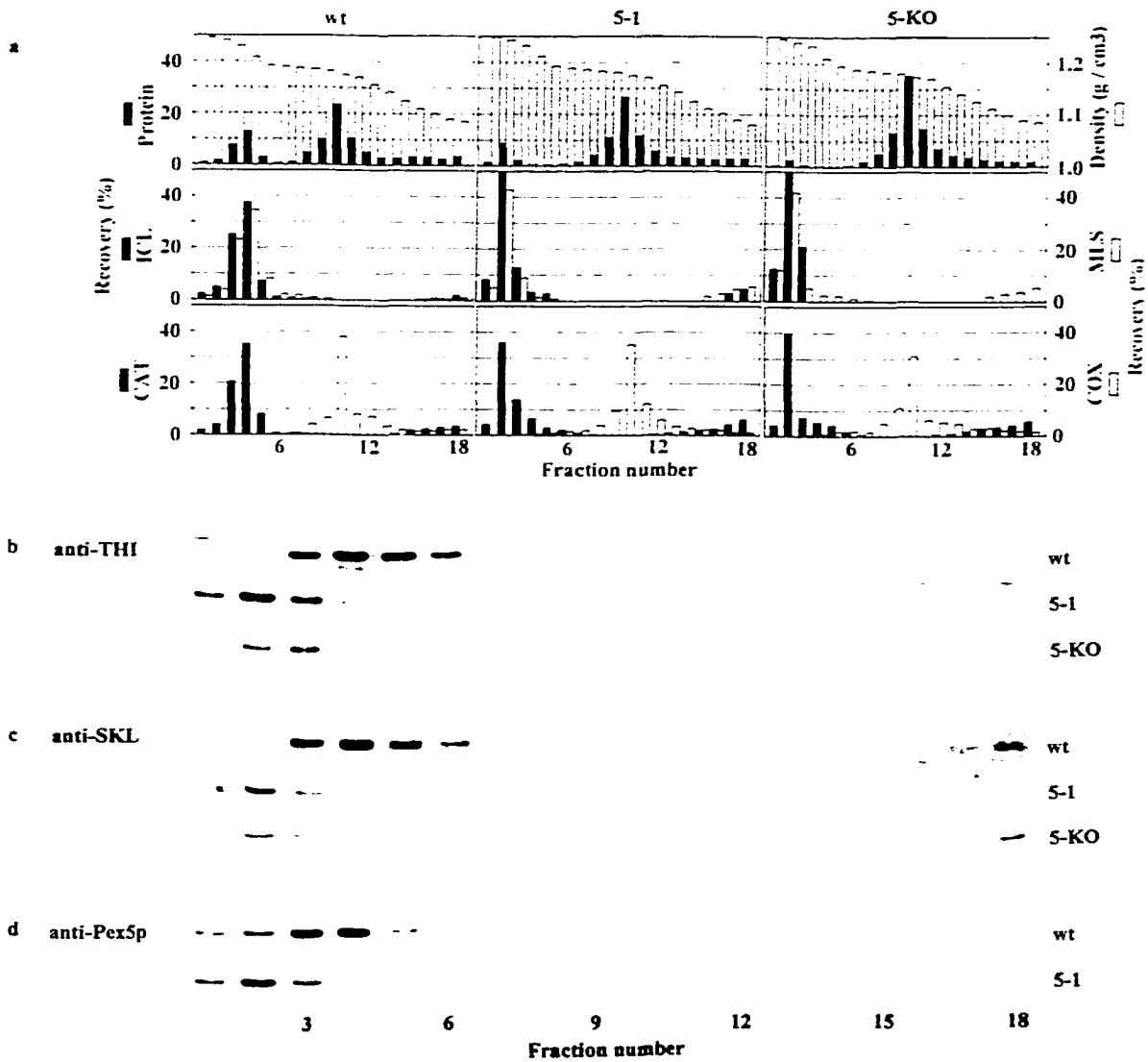


Figure 3-12. Purification of peroxisomes and localization of proteins. Wild-type (*wt*), *pex5-1* (*5-1*) and *pex5-KO* (*5-KO*) strains were grown as described in the legend to Figure 3-11. Fractions enriched for peroxisomes were isolated as described in Section 2.10.4.2. For each strain 9 mg of protein was loaded onto the gradient. (*Upper panels*) Sucrose density (g/cm^3) and percent recovery of loaded protein and of enzymatic activities in gradient fractions. *COX*, cytochrome *c* oxidase. Other abbreviations as in Figure 3-11. Gradient fractions were analysed by immunoblotting (*lower panels*) with anti-thiolase (*anti-THI*), anti-SKL and rabbit anti-*Yl*Pex5p (*anti-Pex5p*) antibodies. For immunoblotting with anti-thiolase and anti-SKL antibodies, 2% of the volume of each fraction analysed. For immunoblotting with anti-*Yl*Pex5p antibodies, 10% of the volume of each fraction was analysed. *Contributed by Dr. Vladimir Titorenko.*

mutant Pex5 proteins co-migrated with the peak of peroxisomal markers (fraction 4 for wild-type, fraction 2 for *pex5-1*; Figure 3-12 D), suggesting that *Yl*Pex5p is also a peroxisomal protein.

The peroxisomal peak fractions were further analysed by sucrose flotation (Heyman *et al.*, 1994). All proteins tested floated out of the most dense sucrose solution and concentrated at the interface between the 60% and 35% sucrose solutions at densities of 1.22 g/cm^3 and 1.25 g/cm^3 for the wild-type and mutant strains, respectively (Figure 3-13). This suggests that the peroxisomal proteins in the wild-type and *pex5* mutant strains colocalized because they were associated with a membrane-bound compartment, and not because of the formation of aggregates.

3.8 A subset of peroxisome-associated proteins is translocated into the peroxisomal matrix in *pex5* mutant cells

The targeting and translocation of various peroxisomal matrix proteins were investigated. Immunoblot analysis of subcellular fractions revealed that thiolase is present in the organelle-enriched 20KgP fraction of the wild-type, *pex5-1* and *pex5-KO* strains (Figure 3-14 a). However, the targeting of thiolase to peroxisomes is less efficient in the mutant strains than in the wild-type strain, since a greater portion of the thiolase in the *pex5-1* and *pex5-KO* mutant cells was found in the 20KgS fraction, compared to that found in the 20KgS fraction from wild-type cells. The amount of thiolase per mg of peroxisomal protein is roughly the same for the three strains (Figure 3-14 b, *lanes PER*). When peroxisomes from the wild-type and *pex5* mutant strains were lysed with Ti8 buffer and subjected to

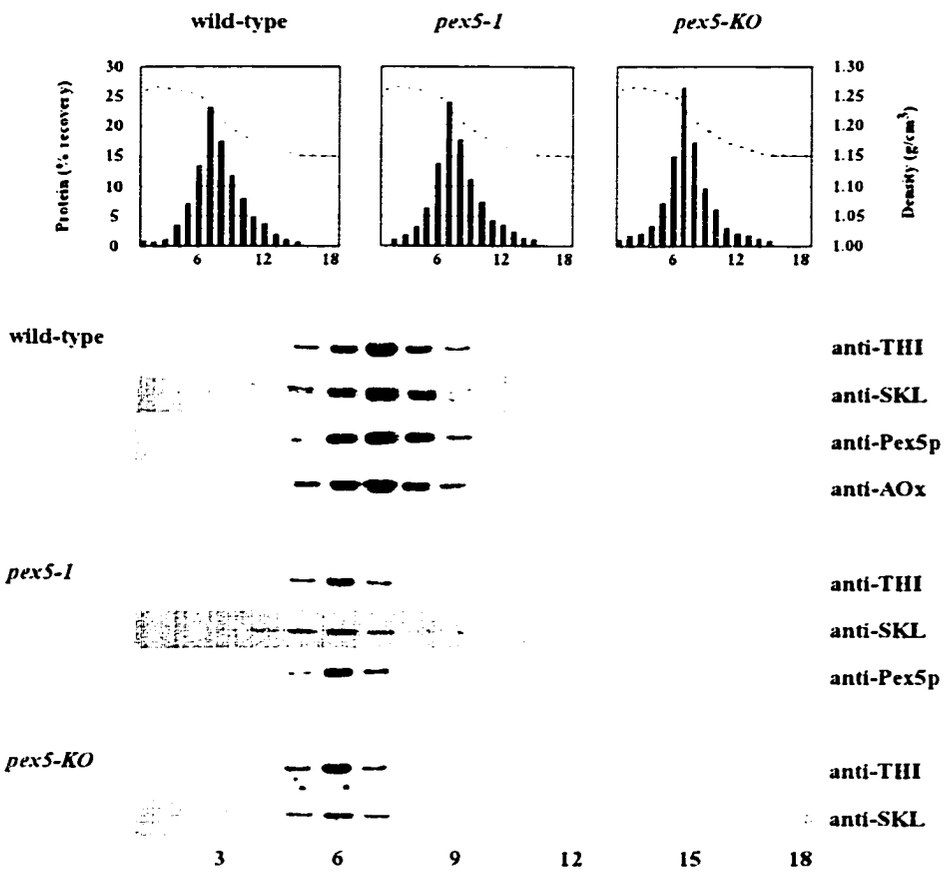
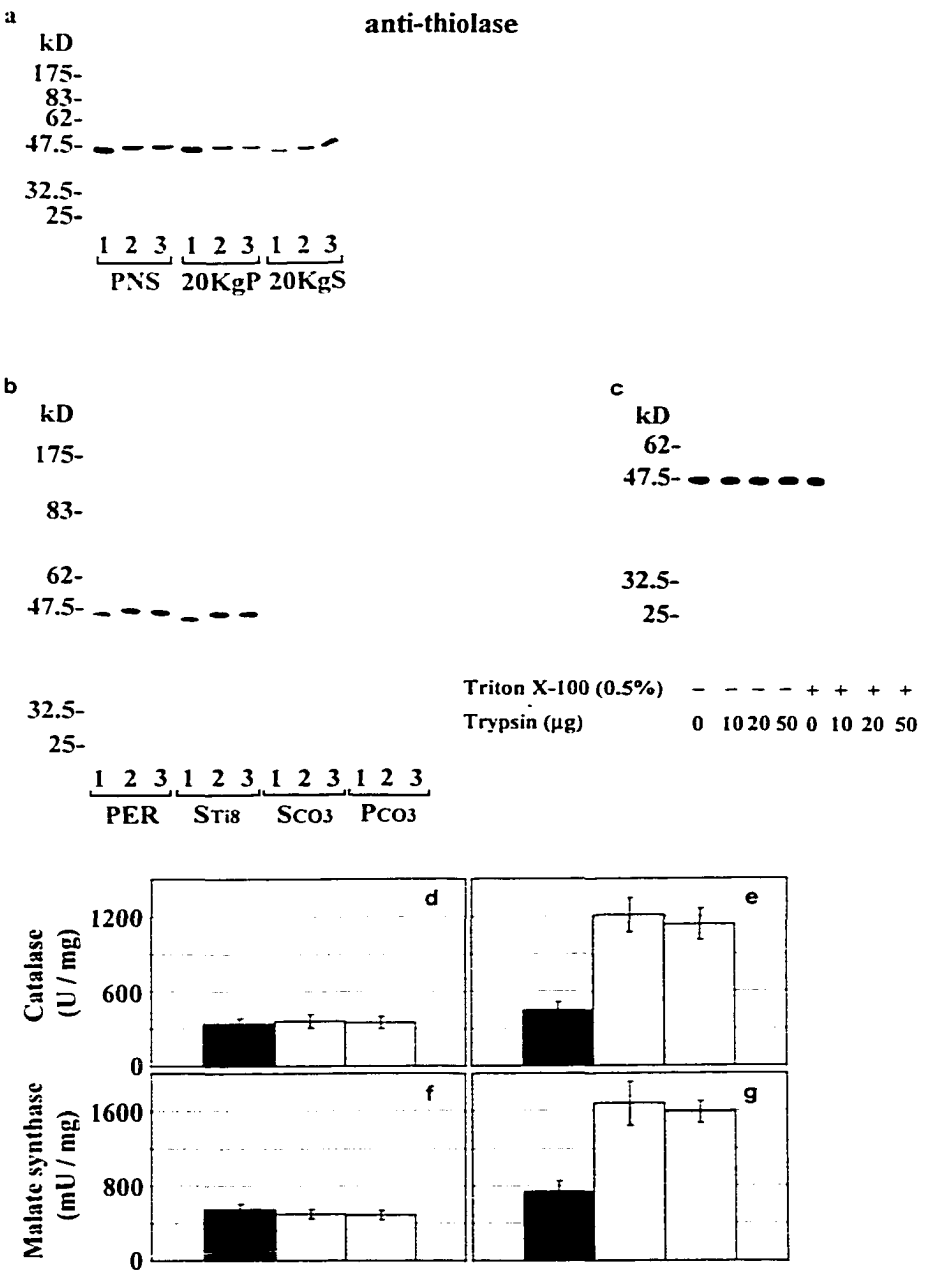


Figure 3-13. Sucrose flotation analysis. 400 µL of peak peroxisomal fractions from isopycnic centrifugation (fraction 4 for wild-type, fraction 2 from *pex5-1* and *pex5-KO*) were overlaid successively with 60% and 35% (w/v) sucrose and subjected to centrifugation as described in Section 2.10.4.3. Fractions were analysed for density (*upper panels, circles*), protein concentration (*upper panels, bars*) and were subjected to SDS-PAGE and immunoblotting (*lower panels*) with anti-thiolase (*anti-THI*), anti-SKL (*anti-SKL*), anti-*Yl*Pex5p (*anti-Pex5p*) and anti-acyl-CoA oxidase (*anti-AOx*) antibodies. *Contributed by Dr. Vladimir Titorenko.*

Figure 3-14. Thiolase, catalase and malate synthase are imported into the peroxisomal matrix of *pex5* mutant cells, but thiolase processing does not occur. (*a*) Thiolase distribution in subcellular fractions. Equal fractions (0.2% of the total volume) of the post nuclear supernatant (*PNS*), 20KgP and 20KgS from the wild-type (*lanes 1*), *pex5-1* (*lanes 2*) and *pex5-KO* (*lanes 3*) were analysed by immunoblotting with anti-thiolase antibodies. (*b*) Thiolase distribution in peroxisomal subfractions. Whole peroxisomes (20 μg of protein) were subjected to subfractionation as described in Section 2.10.4.4. *PER*, whole peroxisomes (20 μg); S_{Ti8}, 200,000 x *g* supernatant recovered after peroxisome lysis with Ti8 buffer; S_{CO3} and P_{CO3}, 200,000 x *g* supernatant and pellet, respectively, after the treatment of the 200,000 x *g* pellet from Ti8 lysis (P_{Ti8}) with 0.1 M Na_2CO_3 (pH 11). Lanes are numbered as for panel a. (*c*) Protease protection analysis. The 20KgP fraction (240 μg) of the *pex5-KO* strain was incubated with 0, 10, 20 or 50 μg of trypsin in the absence (-) or presence (+) of 0.5% (v/v) Triton X-100 for 40 min on ice. Reactions were terminated by the addition of TCA to 10%. Equal fractions of the samples were analysed by immunoblotting with anti-thiolase antibodies. (*d-g*) Specific activities of catalase (*d and e*) and malate synthase (*f and g*) in the wild-type (*solid bar*), *pex5-1* (*stippled bar*) and *pex5-KO* (*open bar*) strains. Activities were measured in whole peroxisomes (*d and f*) and in the S_{Ti8} enriched for peroxisomal matrix proteins (*e and g*). The values reported are the means ± the standard deviation for three independent experiments. *Contributed by Dr. Vladimir Titorenko.*



centrifugation at 200,000 x *g* to pellet peroxisomal membranes, thiolase was found exclusively in the supernatant fraction enriched for peroxisomal matrix proteins (Figure 3-14 b, *lanes* S_{Tis}). The localization of thiolase in the matrix of peroxisomes from *pex5-KO* cells was further suggested by protease protection analysis. Thiolase was resistant to trypsin digestion in the absence of detergent, but was sensitive to protease digestion when detergent was present (Figure 3-14 c). Peroxisome-associated thiolase was protected from trypsin digestion only under conditions where the peroxisomal membrane was intact, implying that in *pex5-KO* cells, thiolase is present in the matrix of peroxisomes. Taken together, these data suggest that while the targeting of thiolase is somewhat inefficient in cells with mutations in the *PEX5* gene, thiolase that reaches the peroxisomal surface is imported correctly into the matrix in these strains. It is also noteworthy that in *pex5* mutant cells, the peroxisome-associated thiolase is in its larger, precursor form. This implies that the processing of thiolase is not essential for its import into peroxisomes, as has been previously noted for PTS2-containing proteins with cleavable presequences (Gietl *et al.*, 1994; Motley *et al.*, 1994). This further suggests that the putative processing protease is either inactive or not correctly localized in *pex5* mutant strains.

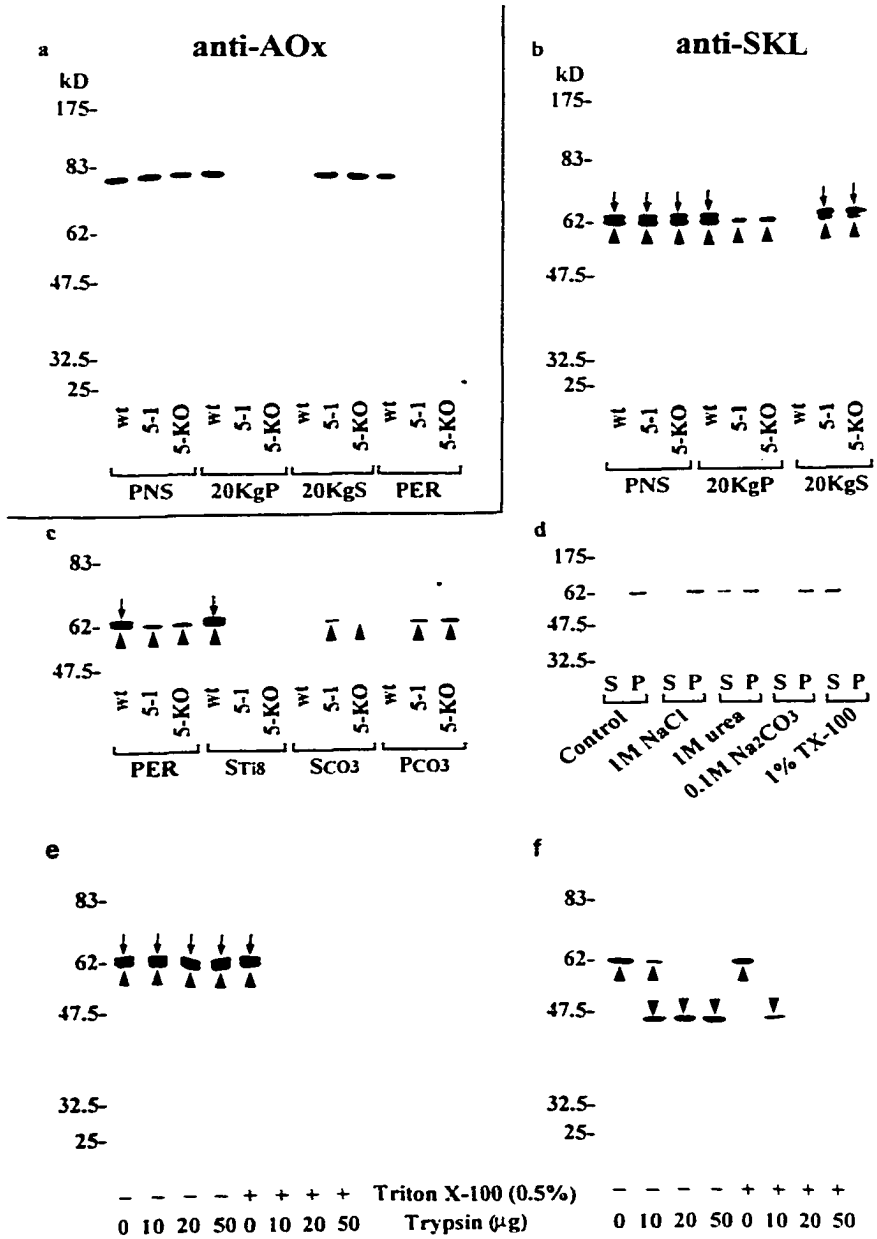
The activities of catalase and malate synthase were measured in subcellular and subperoxisomal fractions. As in the case of thiolase, the targeting of these proteins to peroxisomes is inefficient in the *pex5* mutant strains (Figure 3-11, *middle and lower panels, CAT and MLS*). The specific activities of both enzymes in the total peroxisomal fraction were approximately the same in wild-type and *pex5* mutant cells (Figure 3-14 d and f), but the specific activities in the peroxisomal matrix fractions were 2- to 3-fold higher in the *pex5-1*

and *pex5-KO* strains than in the wild-type strain (Figure 3-14 e and g). This suggests that in cells harbouring *pex5* mutations, the catalase and malate synthase that are targeted to peroxisomes are able to be translocated into the matrix. However, the enrichment of these two enzymes in the matrix fraction suggests that not all proteins that reach the peroxisomal membrane are able to be imported into the matrix. This import defect leads to a selective enrichment of some proteins, such as catalase and malate synthase, in the peroxisomal matrix.

3.9 Many proteins targeted to peroxisomes are not correctly imported into peroxisomes in cells with *PEX5* mutations

An unidentified anti-SKL reactive protein with an apparent molecular mass of 62 kD, p62, was partially localized to the 20KgP in *pex5* mutant cells, but was found exclusively in the 20KgP of wild-type cells (Figure 3-15 b, *upward pointing arrowheads*). This protein was found in peroxisomes of the *pex5* mutant and wild-type strains at approximately the same levels when equal amounts of peroxisomal proteins were compared (Figure 3-15 c, *lanes PER*). However, while p62 was found exclusively in the matrix-enriched fraction of peroxisomes from wild-type cells lysed with Ti8 buffer (Figure 3-15 c, S_{Ti8}, *lane wt*), in *pex5* mutant cells p62 localized exclusively to the membrane-enriched fractions (Figure 3-15 c, compare S_{CO3} *and* P_{CO3}, *lanes 32-1 and 32-KO*). Some p62 in the *pex5* mutant strains seemed to be peripherally associated with the membrane, since it could be extracted with carbonate (Figure 3-15 C, S_{CO3}, *lanes 32-1 and 32-KO*), but the majority of the peroxisome-associated p62 in these strains was resistant to carbonate extraction (Figure 3-15 c, P_{CO3}, *lanes 32-1 and 32-KO*), and therefore behaved as an integral membrane protein (Fujiki *et al.*, 1982). Some

Figure 3-15. Mutations in the *PEX5* gene prevent the targeting of acyl-CoA oxidase and a 64-kD anti-SKL-reactive polypeptide to peroxisomes, and prevent the complete translocation, but not the targeting, of a 62-kD anti-SKL-reactive polypeptide into peroxisomes. (*a and b*) Distribution of acyl-CoA oxidase and anti-SKL-reactive polypeptides in subcellular fractions. Equal portions of the post-nuclear supernatant (*PNS*), 20 kgP and 20KgS fractions and 10 μg of peroxisomes (*PER*) from the wild-type (*wt*), *pex5-1*(*5-1*) and *pex5-KO* (*5-KO*) strains were analysed by immunoblotting with anti-acyl-CoA oxidase (*anti-AOx*) (*a*) or anti-SKL (*b*) antibodies. (*c*) Distribution of anti-SKL-reactive polypeptides in peroxisomal subfractions. *PER* lanes contained 20 μg of purified peroxisomes. Peroxisomal subfractions were isolated from 20 μg of purified peroxisomes as described in Figure 3-14 b. Immunoblot analysis was performed with anti-SKL antibodies. (*d*) The P_{Ti8} from the *pex5-KO* strain was resuspended in Ti8 buffer, and equal aliquots were treated with one of 1 M NaCl, 1 M urea, 0.1 M Na_2CO_3 or 1% (w/v) Triton X-100 (*TX-100*). The control was treated with Ti8 buffer alone. After incubation on ice for 45 min, samples were separated by centrifugation at 200,000 x *g* for 1 h at 4°C into supernatant (*S*) and pellet (*P*) fractions. Samples were immunoblotted with anti-SKL antibodies. (*e and f*) Protease protection analysis of anti-SKL reactive proteins in peroxisomes of wild-type (*e*) and *pex5-KO* (*f*) strains. Protease protection was carried out as described in the legend to Figure 3-14 d. *Arrow*, 64-kD anti-SKL-reactive polypeptide; *upward pointing arrowhead*, 62-kD anti-SKL-reactive polypeptide; *downward pointing arrowhead*, 45-kD trypsin-resistant fragment. *Contributed by Dr. Vladimir Titorenko.*



of the peroxisomal p62 in the *pex5-KO* strain had a strong association with the peroxisomal membrane, since it was at least partially resisitant to extraction by agents that specifically solubilize peripheral membrane proteins (1 M NaCl, 1 M urea), but was almost completely released to the soluble fraction when the membrane was disrupted with Triton X-100 (Figure 3-15 d).

Protease protection analysis of p62 from wild-type cells revealed that this protein was resistant to trypsin digestion in the absence of detergent, but was sensitive to the protease upon the addition of Triton X-100 (Figure 3-15 e, *upward pointing arrowheads*). This is a typical finding for a matrix protein, since it is shielded from the protease by an intact peroxisomal membrane. In contrast, the peroxisome-associated p62 from *pex5-KO* cells was sensitive to protease digestion even in the absence of detergent (Figure 3-15 f, *upward pointing arrowheads*). An anti-SKL-reactive polypeptide that ran with an apparent molecular mass of ~45 kD was trypsin-resistant in the absence of detergent, but was degraded when Triton X-100 was added (Figure 3-15 f, *downward pointing arrowheads*). These results suggest that in *pex5* mutant cells, p62 is (inefficiently) targeted to peroxisomes, where it becomes tightly associated with the peroxisomal membrane in such a way that the 45-kD anti-SKL reactive portion is protected and the remaining 17-kD portion is at least partially exposed to the cytoplasm. One scenario consistent with these data is that in *pex5* mutant cells, the majority of peroxisome-associated p62 is trapped in the membrane in an intermediate stage of translocation, with its C-terminus in the peroxisomal matrix.

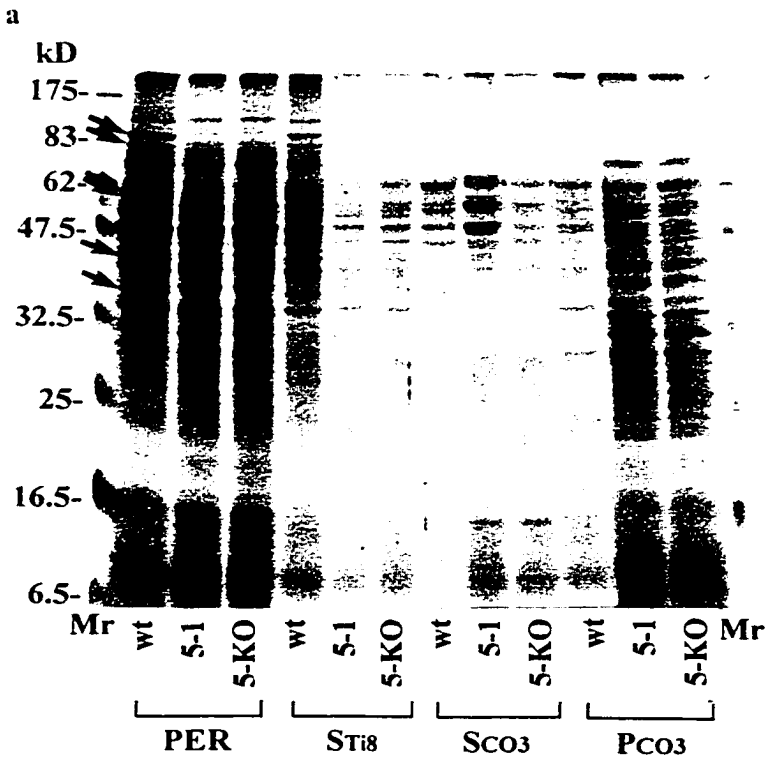
Peroxisomes and peroxisomal subfractions were also analysed by SDS-PAGE and Coomassie Blue staining. The protein profiles of whole peroxisomes from wild-type and *pex5*

mutant cells were very similar (Figure 3-16 a, *lanes PER*), indicating that, with a few notable exceptions (Figure 3-16 a, *arrows*), most proteins are correctly targeted to peroxisomes in *pex5* mutant cells. In subfractions of peroxisomes from wild-type cells, most proteins were found in the matrix-enriched fraction (Figure 3-16 a, S_{Ti8}, *lane wt*). In contrast, most peroxisomal proteins from *pex5* mutant cells were tightly associated with the peroxisomal membrane (Figure 3-16 a, P_{CO3}, *lanes 32-1 and 32-KO*). Many of the proteins in the P_{Ti8} were not extractable with 1 M NaCl and 1 M urea that specifically release peripheral membrane proteins, and were only solubilized when the membrane was disrupted with 1% Triton X-100 (Figure 3-16 b). These data suggest that most proteins are targeted to peroxisomes in a *Yl*Pex5p-independent manner, but they are translocated into peroxisomes in a *Yl*Pex5p-dependent manner. In *pex5-1* and *pex5-KO* cells, the majority of proteins targeted to peroxisomes accumulate at the membrane because they are not able to penetrate fully into the matrix.

3.10 *PEX5* mutations prevent the targeting of a subset of proteins to peroxisomes

The 3-hydroxyacyl-CoA dehydrogenase activity of the multifunctional β-oxidation enzyme (MFE2; J.J. Smith, T.W. Brown, G.A. Eitzen and R.A. Rachubinski, manuscript submitted) was found exclusively in the 20KgP fraction of wild-type cells, whereas MFE2 activity was completely mislocalized to the 20KgS in *pex5-1* and *pex5-KO* cells (Figure 3-11, *middle and bottom panels, HAD*). This suggests that the targeting of MFE2 to peroxisomes is abolished in *pex5* mutant strains. Immunoblotting of subcellular fractions revealed that the same was true for acyl-CoA oxidase (Figure 3-15 a) and p64, an anti-SKL reactive protein

Figure 3-16. Mutations in the *PEX5* gene prevent the translocation of most peroxisomal matrix proteins across the peroxisomal membrane. (*a*) Protein profiles of purified peroxisomes (*PER*, 60 μg of protein) and peroxisomal subfractions (S_{Ti8}, S_{CO3}, P_{CO3}) isolated from 60 μg of purified peroxisomes of wild-type, *pex5-1* and *pex5-KO* strains. (*b*) The P_{Ti8} of the *pex5-KO* strain was resuspended in Ti8 buffer and treated with 1% sodium deoxycholate (*DOC*), 1 M NaCl, 1 M urea or 1% (v/v) Triton X-100 as described in the legend to Figure 3-15 d. The control was treated with Ti8 buffer alone. All samples were subjected to SDS-PAGE and stained with Coomassie Blue. Abbreviations are as in Figure 3-15. *Contributed by Dr. Vladimir Titorenko.*



with an apparent molecular mass of ~64 kD (Figure 3-15 b, *arrows*) normally present in the peroxisomal matrix (Figure 3-15 c and e, *arrows*). p64 is apparently isocitrate lyase (Eitzen, 1997). Although immunoblotting did not detect p64 in the 20KgP or peroxisomes of *pex5* mutant cells (Figure 3-15 b and c, *arrows*), there is a small amount of isocitrate lyase activity detectable in these fractions (Figure 3-11, *lower panels, ICL*; Figure 3-12 *upper panels, ICL*), but the peroxisome-associated isocitrate lyase activity in *pex5* mutant cells did not exceed 4% of the specific activity in the peroxisomes of wild-type cells.

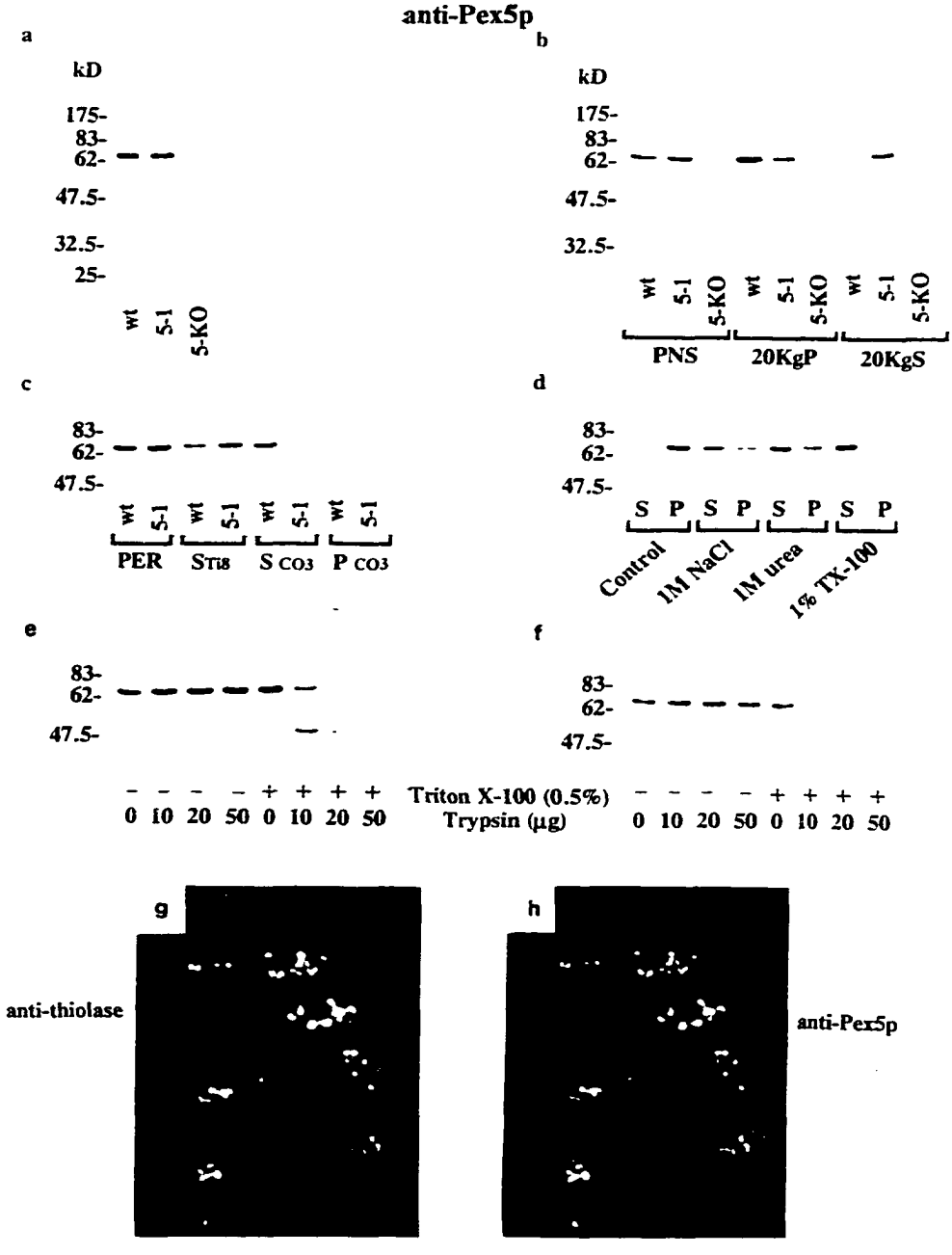
Taken together, these data suggest that the transport of different peroxisomal proteins is affected to varying degrees in *pex5* mutant cells: i) thiolase, catalase and malate synthase can be both targeted to, and translocated across, the peroxisomal membrane (Figure 3-14); ii) p62 and the majority of peroxisome-associated proteins, can be targeted to the peroxisomal membrane, but their import into the matrix is blocked (Figure 3-15 b-f and Figure 3-16, respectively); iii) there is a severe impairment in the targeting to peroxisomes of MFE2, acyl-CoA oxidase and p64/isocitrate lyase (Figure 3-11, *middle and bottom panels*, Figure 3-15 a, and Figure 3-15 b and c, respectively).

3.11 *Yl*Pex5p is an intraperoxisomal protein

*Yl*Pex5p and *Yl*pex5-1p (the protein encoded by the *pex5-1* allele) reached the same steady-state level in wild-type and *pex5-1* cells (Figure 3-17 a). *Yl*pex5-1p has the same apparent electrophoretic mobility as *Yl*Pex5p, suggesting that the mutation in the *pex5-1* allele does not result in a truncated Pex5 polypeptide.

In subcellular fractionation experiments, *Yl*Pex5p was detectable only in the 20KgP

Figure 3-17. *Yl*Pex5p is an intraperoxisomal protein associated with both the inner membrane surface and matrix of wild-type peroxisomes. (*a*) Immunoblot analysis of whole cell extracts (40 μg of protein) probed with anti-*Yl*Pex5p antibodies. Cells were grown in YPBO for 9h. (*b*) Distribution of *Yl*Pex5p in subcellular fractions. Equal portions (0.2% of total volume) of the post-nuclear supernatant (*PNS*), 20KgP and 20KgS fractions were analysed by immunoblotting with anti-*Yl*Pex5p antibodies. (*c*) Distribution of *Yl*Pex5p in peroxisomal subfractions of wild-type and *pex5-1* strains. Purified peroxisomes (*PER*, 60 μg of protein) and peroxisomal subfractions (S_{Ti8}, S_{CO3} *and* P_{CO3}) prepared from an equal amount of starting material were analysed by immunoblotting with anti-*Yl*Pex5p antibodies. (*d*) The P_{Ti8} of the wild-type strain was resuspended in Ti8 buffer and extracted with various agents and divided into supernatant (*S*) and pellet (*P*) as described in the legend to Figure 3-15 d. The control was treated with Ti8 buffer alone. Equal portions of supernatant and pellet were analysed by immunoblotting with anti-*Yl*Pex5p antibodies. (*e and f*) Protease protection analysis. The 20KgP fractions of the wild-type (*e*) and *pex5-1* (*f*) strains were subjected to trypsin digestion as in Figure 3-14 d and analysed by immunoblotting with anti-*Yl*Pex5p antibodies. (*g and h*) Double-labelling, indirect immunofluorescence analysis of wild-type cells using guinea pig anti-thiolase (*g*) and rabbit anti-*Yl*Pex5p (*h*) primary antibodies. Primary antibodies were detected with rhodamine-conjugated donkey anti-guinea pig IgG (*g*) and fluorescein-conjugated goat anti-rabbit IgG (*h*) secondary antibodies. *Contributed by Dr. Vladimir Titorenko.*



of wild-type cells (Figure 3-17 b, *lanes wt*), whereas *Yl*pex5-1p (Figure 3-17 b, *lanes 32-1)* was found in both the 20KgP and the 20KgS of *pex5-1* cells. However, the amount of Pex5 protein per microgram of purified peroxisomes from the wild-type and *pex5-1* strains was approximately the same (Figure 3-17 c, *lanes PER*). When peroxisomes were lysed with Ti8 buffer, ~35% of the *Yl*Pex5p from wild-type cells was recovered in the matrix-enriched fraction (Figure 3-17 c, S_{Ti8}, *lane wt*), while ~70% was recovered in the membrane-enriched fractions (Figure 3-17 c, S_{CO3} *and* P_{CO3}, *lanes wt*). The membrane-associated *Yl*Pex5p could be completely extracted by carbonate treatment (Figure 3-17 c, S_{CO3} to P_{CO3}, *lanes wt*), thus suggesting that this pool of *Yl*Pex5p was peripherally associated with the peroxisomal membrane. The association of *Yl*Pex5p with the membrane was quite tight, since it was partially resistant to extraction with salt or urea, and was only completely solubilized when the membrane was disrupted with detergent (Figure 3-17 d) or stripped with carbonate (Figure 3-17 c, *lanes wt*). In contrast, all peroxisomal *Yl*pex5-1p was found in the organellar matrix fraction (Figure 3-17 c, S_{Ti8}, *lane 32-1*); this protein was not detected in the fractions containing membrane-associated proteins (Figure 3-17 c, S_{CO3} and P_{CO3}, *lanes 32-1*).

Protease protection experiments were performed with purified peroxisomes from wild-type and *pex5-1* cells. *Yl*Pex5p and *Yl*pex5-1p were not digested by trypsin in the absence of detergent (Figure 3-17 e and f, *lanes* -) but were sensitive to the protease when 0.5% Triton X-100 was present (Figure 3-17 e and f, *lanes* +). These results suggest that *Yl*Pex5p and *Yl*pex5-1p are located inside peroxisomes, since these proteins were only digested under conditions in which the integrity of the peroxisomal membrane is compromised. In the presence of detergent, *Yl*pex5-1p was completely degraded by the addition of 10 μg of

trypsin; under the same conditions, *Yl*Pex5p was partially resistant to degradation, as evidenced by the presence of a proteolytic fragment that ran with an apparent molecular mass of ~52 kD (Figure 3-17, compare *panel f, lane +10* to *panel e, lane +10*). The partial protection of *Yl*Pex5p from wild-type cells could have arisen from the association of a fraction of the protein with the peroxisomal membrane (see Figure 3-17 c, *lanes wt*), thus reducing the accessibility of the protease to the peroxin. In contrast, *Yl*pex5-1p showed no association with the peroxisomal membrane (Figure 3-17 c, *lanes 32-1*), which could account for its greater sensitivity to the protease.

The localization of *Yl*Pex5p in peroxisomes was confirmed in the wild-type strain by double labelling, indirect immunofluorescence microscopy using anti-thiolase (Figure 3-17 g) and anti-*Yl*Pex5p (Figure 3-17 h) antibodies. The punctate patterns, which are characteristic of peroxisomal staining and are superimposable for all cells, demonstrate that thiolase and *Yl*Pex5p are localized to the same compartment.

3.12 *Yl*Pex5p associates with anti-SKL-reactive proteins *in vivo*

Immunoprecipitation experiments were performed using peroxisomal matrix proteins of wild-type and *pex5-1* cells. After incubating the S_{Ti8} fraction with immobilized anti-SKL or anti-*Yl*Pex5p antibodies, the proteins from the flow-throughs, washes and eluates were analysed by SDS-PAGE and immunoblotting with various antisera. Much of both p62 and p64 could be co-immunoprecipitated with antibodies to *Yl*Pex5p from the peroxisomal matrix fraction of wild-type cells; similarly, most of the *Yl*Pex5p could be co-immunoprecipitated with anti-SKL antibodies (Figure 3-18 a). This suggests that *Yl*Pex5p associates with anti-

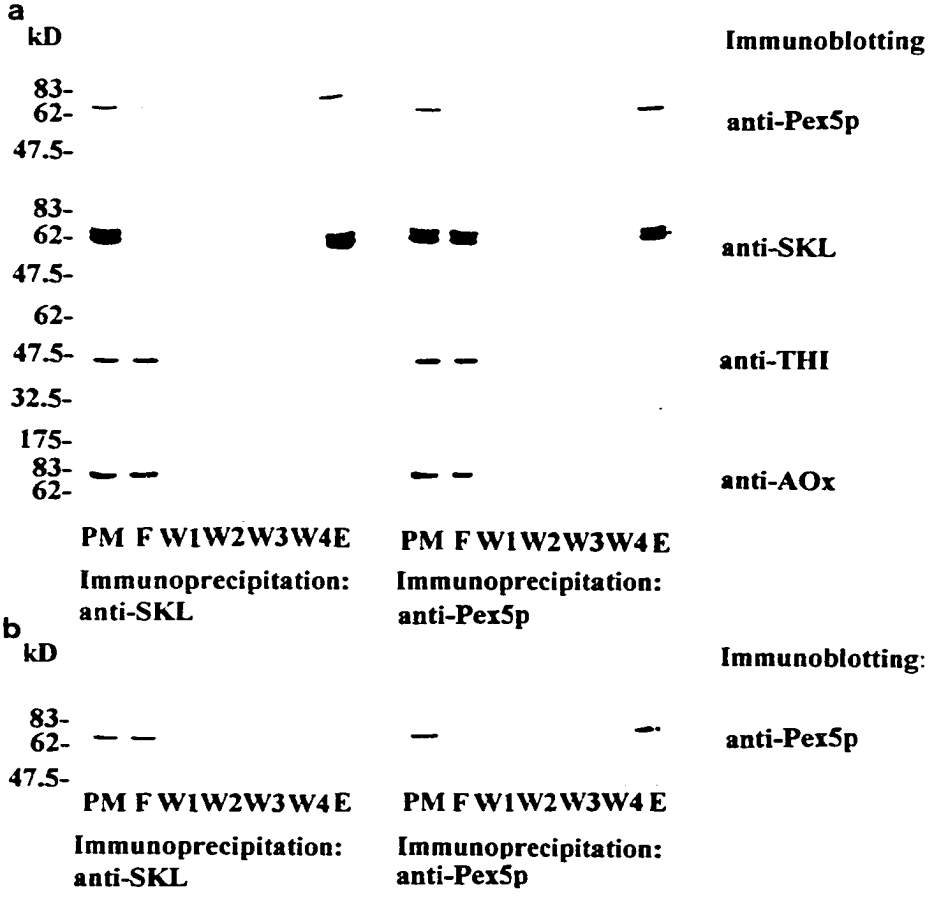


Figure 3-18. *Yl*Pex5p and anti-SKL-reactive proteins form a complex in the peroxisomal matrix of wild-type cells. Matrix proteins (S_{Ti8} from peroxisomes of the wild-type (*a*) or *pex5-1* mutant (*b*) were subjected to native immunoprecipitation (Section 2.10.4.6.1) with anti-SKL (*panels at left*) or anti-*Yl*Pex5p (anti-Pex5p; *panels at right*) antibodies linked to protein A-Sepharose. *PM*, peroxisomal matrix proteins (20 μg, equivalent to amount applied to column); *F*, proteins recovered in flow-through; *W1-W4*, proteins recovered in washes; *E*, proteins eluted with 100 mM glycine (pH 2.8). Proteins were subsequently analysed by immunoblotting with anti-*Yl*Pex5p, anti-SKL, anti-thiolase (*anti-THI*) and anti-acyl-CoA oxidase (*anti-AOx*) antibodies. *Contributed by Dr. Vladimir Titorenko.*

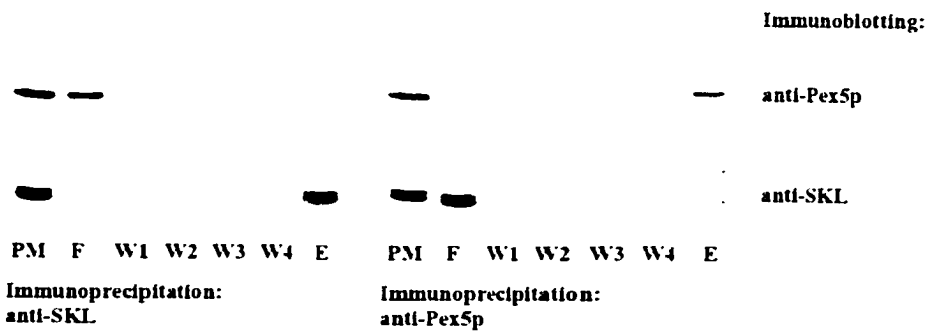


Figure 3-19. Anti-*Yl*Pex5p and anti-SKL antibodies specifically immunoprecipitate only their target proteins under denaturing conditions. Matrix proteins (S_{Ti8} from peroxisomes of the wild-type strain were subject to denaturing immunoprecipitation (Section 2.10.4.6.2) with anti-SKL(*panels at left*) or anti-*Yl*Pex5p (*anti-Pex5p*) antibodies linked to protein A-Sepharose. Proteins were subsequently analysed by immunoblotting with anti-*Yl*Pex5p and anti-SKL antibodies. *Symbols* are as for Figure 3-18. *Contributed by Dr. Vladimir Titorenko.*

SKL-reactive proteins in the matrix of wild-type peroxisomes. The interaction of *Yl*Pex5p and the anti-SKL reactive proteins was specific, since 1) neither anti-Pex5p nor anti-SKL antibodies could co-immunoprecipitate thiolase or acyl-CoA oxidase (Figure 3-18 a); 2) under native immunoprecipitation conditions, anti-SKL antibodies could not co-immunoprecipitate *Yl*pex5-1p from the peroxisomal matrix fraction of *pex5-1* cells (Figure 3-18 b) which contains no anti-SKL reactive proteins (Figure 3-15 c); 3) anti-SKL and anti-*Yl*Pex5p antibodies could only precipitate their target proteins from wild-type peroxisomal matrix proteins denatured with SDS (Figure 3-19). This experiment does not discriminate between a direct or indirect interaction between *Yl*Pex5p and the anti-SKL-reactive proteins.

In the *pex5-1* mutant, p62 appears to be stuck in the peroxisomal membrane with its anti-SKL-reactive portion facing the matrix (Figure 3-15 f). *Yl*pex5-1p does not associate with the membrane (Figure 3-17 c), and therefore does not complex with anti-SKL-reactive proteins. However, these experiments do not demonstrate whether a failure of *Yl*pex5-1p to interact with anti-SKL-reactive proteins or a failure to associate with the inner face of the peroxisomal membrane is the primary defect in these cells.

3.13 Discussion

This chapter documents the identification of the *pex5-1* mutant, its morphological and biochemical characterization, the cloning of the *PEX5* gene and an analysis of its predicted product, *Yl*Pex5p, as well as preliminary elucidation of its biochemical function.

3.13.1 YlPex5p is an intraorganellar member of a conserved family of peroxisomal protein import factors

The sequence of *Yl*Pex5p is highly similar to those of members of the Pex5 family of peroxins, especially in the characteristic C-terminal TPR domain (Appendix 1). The first Pex5p family member to be identified was from the yeast *P. pastoris* (McCollum *et al.*, 1993). This peroxin was subsequently found in other yeasts (*S. cerevisiae* (van der Leij *et al.*, 1993), *H. polymorpha* (Nuttley *et al.*, 1995; van der Klei *et al.*, 1995), mammals (human (Fransen *et al.*, 1995; Dodt *et al.*, 1995; Wiemer *et al.*, 1995), mouse (Baes *et al.*, 1997), Chinese hamster (Otera *et al.*, 1998), guinea pig (SwissProt O70525)), plants (watermelon (Wimmer *et al.*, 1998), tobacco (Kragler *et al.*, 1998), *Arabidopsis thaliana* (Brickner *et al.*, 1998)) and a kinetoplastid (*Trypanosoma brucei* (de Walque *et al*, 1999)). Genome sequencing projects have also identified putative orthologues in an insect (*Drosophila melanogaster* (SwissProt O46085)), a nematode (*Caenorhabditis elegans* (SwissProt Q18426)) and possibly in the fission yeast *Schizosaccharomyces pombe* (EMBL AL034342). For several of these proteins, the TPR domain has been demonstrated to be essential for their function as PTS1-binding proteins (McCollum *et al.*, 1993; Brocard *et al.*, 1994; Terlecky *et al.*, 1995; Fransen *et al.*, 1995; Dodt *et al.*, 1995; Wiemer *et al.*, 1995; Kragler *et al.*, 1998).

*Yl*Pex5p is detected exclusively in the matrix of peroxisomes, where it is primarily associated with the inner face of the peroxisomal membrane (Figure 3-17 b, c and g). The data presented in this study are consistent with *Yl*Pex5p being an intraperoxisomal component of the matrix protein translocation machinery. Other Pex5 family members have been reported to have different localizations, which has led to various hypotheses concerning their

mechanism of action. Pex5 proteins found to be associated with the outer side of the peroxisomal membrane (McCollum *et al.*, 1993; Fransen *et al.*, 1995; Terlecky *et al.*, 1995) have been suggested to be static PTS1 receptors. Family members that were found to be primarily cytosolic (van der Leij *et al.*, 1993; Dodt *et al.*, 1995; Wiemer *et al.*, 1995; Elgersma *et al.*, 1996a; Wimmer *et al.*, 1998; de Walque *et al.*, 1999) or present to a significant extent in both the cytosol and peroxisomes (van der Klei *et al.*, 1995) have been proposed to be shuttling receptors.

The same controversy exists in the Pex7 family of PTS2 receptors. Zhang and Lazarow (1995; 1996) reported that *S. cerevisiae* Pex7p is exclusively localized to the matrix of peroxisomes. However, the group of Kunau has reported *Sc*Pex7p to be primarily cytosolic (Marzioch *et al.*, 1994; Rehling *et al.*, 1996). In both instances, epitope tags at the C- and N-termini, respectively, may have influenced *Sc*Pex7p localization and/or function. *P. pastoris* Pex7p (unmodified) was found to be primarily cytosolic (Elgersma *et al.*, 1998).

As mentioned in Chapter 1, a possible explanation for the variation in Pex5p (and Pex7p) localizations found in different species, or even in the same species, is that all these proteins are in fact receptors that shuttle between the cytosol, peroxisomal membrane and peroxisomal matrix. Under different cellular conditions (for example, the stage of peroxisome proliferation), the relative amounts of the receptors in different compartments may change (Rachubinski and Subramani, 1995).

Alternatively, PTS1-binding TPR proteins might be organized as an integrated system for the import of PTS1-targeted proteins. In this scenario, individual members of the Pex5 family would be localized to different subcellular compartments (or exist as separate soluble

and membrane-bound isoforms), with import substrates being passed from one TPR protein to the next. This hypothesis would predict that multiple PTS1-binding TPR proteins should be present in a given species. Recently, two distinct but highly similar *PEX5* cDNAs have been identified in tobacco (Kragler *et al.*, 1998). It will be interesting to learn if the two predicted proteins are redundant, or if they have unique binding specificities, localizations, and/or regulation. The *S. cerevisiae* genome also encodes a second potential protein with a sequence highly similar to known Pex5 family members (SwissProt accession number Q04364), but there are no reports of its possible role in peroxisome biogenesis.

It also may be the case that over the course of evolution different species have adopted unique strategies for optimizing peroxisomal protein import, and that what may be true for one (group of) species may not be true for another. There is certainly evidence for species differences in Pex5p function; while PTS1 and PTS2 import pathways are independent in yeast species, the long form of mammalian Pex5 proteins is also essential for import of PTS2-containing proteins (Braverman *et al.*, 1998; Otera *et al.*, 1998).

The *P. pastoris* and *Y. lipolytica* Pex5 peroxins did not demonstrate heterologous functional complementation (Figure 3-10). A negative result of this type could indicate that 1) the two proteins serve different cellular functions; 2) that structural differences prevent them from interacting with other components of the translocation machinery in a heterologous context; or 3) that they have different PTS binding specificities. Interspecies complementation experiments reported to date have entailed expressing foreign *PEX5* genes in a *pex5* mutant of yeast (*P. pastoris*, *S. cerevisiae*, or *H. polymorpha*), and assaying for restored growth on a carbon source requiring peroxisomes for its metabolism (Dodt *et al.*,

1995; Wiemer *et al.*, 1995; Kragler *et al.*, 1998; Wimmer *et al.*, 1998). To date, complete functional complementation using intact, heterologous Pex5 proteins has not been documented. In some cases, this may be explained by the recent report that Pex5 proteins from different organisms preferentially recognize subsets of possible PTS1s, and also have different requirements for the context in which the PTS1 is presented (Lametschwandtner *et al.*, 1998). Therefore, if an essential matrix protein carried a PTS1 (in a context) that was not efficiently recognized by the foreign Pex5p, that matrix protein would not be imported into peroxisomes, thus preventing growth on peroxisome-requiring medium. Taking this into account, possibly a more appropriate experiment to assess the functional equivalence of Pex5 proteins would be to assay for the restoration of import of a protein terminating in SKL, which seems to be universally effective as a PTS1. Greater success has sometimes (Dodt *et al.*, 1995; Wiemer *et al.*, 1995; Kragler *et al.*, 1998), but not always (Wimmer *et al.*, 1998), been achieved with hybrid proteins in which the foreign TPR domain is linked to the host organism's endogenous Pex5p N-terminus, suggesting the importance of the N-terminal region for Pex5p function in some cases.

3.13.2 The importance of YlPex5p for peroxisomal matrix protein import

Most proteins normally found in the peroxisomal matrix are mislocalized to a membrane-enriched fraction in *pex5* mutant strains (Figure 3-16 a). Some of these proteins can be solubilized with agents specific for peripheral membrane proteins, while others are completely extracted only with detergent and therefore behave as would be expected for integral membrane proteins (Figure 3-16 b). p62, the 62-kD anti-SKL-reactive protein, is

found in both peripheral and integral pools of peroxisomal membrane-associated proteins in these strains (Figure 3-15 c). The integral membrane pool of p62 seems to be positioned in the peroxisomal membrane with a 45-kD C-terminal segment facing the matrix, and a 17-kD segment exposed to the cytosol (Figure 3-15 f). These data suggest that p62, and the other carbonate-resistant peroxisomal membrane proteins specific to *pex5* mutant strains, are trapped in the membrane in an intermediate stage of translocation. Therefore most peroxisomal matrix proteins seem to enter peroxisomes via a *Yl*Pex5p-dependent translocation machinery.

*Yl*Pex5p may function to actively pull proteins into the peroxisomal matrix, because 1) *Yl*Pex5p is normally found both in association with the inner peroxisomal membrane and in the peroxisomal matrix (Figure 3-17 c); 2) a portion of the matrix pool of *Yl*Pex5p is present in a complex with anti-SKL-reactive proteins (Figure 3-18 a); and 3) mutations in the *PEX5* gene do not prevent the targeting of proteins to, and the insertion of proteins into, the peroxisomal membrane, but do prevent the complete transit of proteins into the matrix (Figures 3-14, 3-15, 3-16), thus apparently trapping the proteins in a putative translocon. In *pex5* mutant cells, the mislocalized matrix proteins that are peripherally associated with the peroxisomal membrane could represent proteins that have been successfully targeted to the peroxisome, but which are unable to access *Yl*Pex5p-dependent translocons because these translocons are saturated with incompletely translocated proteins that require *Yl*Pex5p to pull them into the matrix. Proteins that are trapped in the membrane but are not recognized by anti-SKL antibodies could be targeted by other variants of the canonical PTS1 motif, or could be proteins that arrived at the membrane by piggybacking with a PTS1-containing protein

(McNew and Goodman, 1994; Elgersma *et al.*, 1996b).

Mutations in the *PEX5* gene result in different effects on the targeting and translocation of individual peroxisomal matrix proteins. The localizations of peroxisomal matrix proteins in *pex5* mutant strains suggest that some proteins can be imported into peroxisomes in a *Yl*Pex5p-independent manner, while others are prevented from doing so at the level of targeting to the peroxisome or translocation across the peroxisomal membrane; 1) thiolase, catalase and malate synthase are targeted (somewhat inefficiently) to peroxisomes; the peroxisome-associated pools of these proteins are translocated normally into the peroxisomal matrix (Figure 3-14). Further experimentation will be required to determine if these three proteins use the same *Yl*Pex5p-independent translocation machinery or if there are multiple alternate import routes. Preliminary evidence indicates that there may be a separate import route for catalase in *Y. lipolytica* (V.I. Titorenko and R.A. Rachubinski, unpublished observations); 2) p62 and the bulk of peroxisomal matrix proteins are also targeted to peroxisomes at a somewhat reduced level, but those proteins that arrive at the peroxisomal membrane are not translocated normally across the bilayer; they remain trapped at the membrane, apparently either partially translocated and stuck in the membrane, or peripherally associated with its cytosolic face (Figures 3-15 b-f, 3-16, respectively); 3) the targeting of acyl-CoA oxidase, p64 and MFE2 to peroxisomes is abolished (Figures 3-15 a, 3-15 b, and 3-11, respectively).

These different effects may be explained if the *Yl*Pex5p-dependent translocation apparatus is served by a cytosolic receptor system (free or membrane-bound) with varying affinities for individual peroxisomal matrix proteins, analogous to the heterooligomeric import

receptor that has been described for mitochondria (Haucke *et al.*, 1996). In this model, p62 and most peroxisomal proteins would have a high affinity for this receptor system (hereafter referred to as high-affinity proteins), allowing these proteins to bind to the peroxisomal membrane more quickly than proteins with low affinity for the receptor system (hereafter referred to as low-affinity proteins). The latter would include acyl-CoA oxidase, p64 and MFE2. Inhibition of translocation through the *Yl*Pex5p-dependent translocation pathway due to loss or mutation of *Yl*Pex5p would lead to the accumulation of high-affinity proteins on the cytoplasmic surface of the peroxisomal membrane. Saturation of membrane binding sites could interfere with further binding of high-affinity proteins (thus leading to their partial mislocalization to the cytosol) and completely abolish the targeting (binding) of low-affinity proteins (causing their complete cytosolic mislocalization).

While thiolase, catalase and malate synthase may be translocated into peroxisomes primarily through a *Yl*Pex5p-independent pathway (or pathways), these proteins might also normally use *Yl*Pex5p-dependent translocons to a minor extent. However, their affinity for the receptor system serving the *Yl*Pex5p-dependent translocons might be relatively low, and like other low-affinity proteins, their targeting (binding) by this route would be abolished in *pex5* mutants. This could account for the partial mislocalization of thiolase, catalase and malate synthase to the cytosol in these strains.

The receptor systems serving the *Yl*Pex5p-independent (and -dependent) translocation machinery are currently unknown. *Y. lipolytica* thiolase, like other peroxisomal thiolases, contains a PTS2 (de Hoop and AB, 1992), and therefore might be recognized by a putative *Y. lipolytica* orthologue of Pex7p, the PTS2 import receptor. The recently characterized *Y.*

lipolytica Pex20p is essential for the oligomerization of thiolase in the cytosol and its import into peroxisomes (Titorenko *et al.*, 1998). *Yl*Pex20p is structurally unrelated to Pex7 proteins, and is able to bind the mature form of thiolase *in vitro*, suggesting that the interaction is independent of the presence of a PTS2. However, it is not known if Pex20p is required only to oligomerize thiolase and maintain it in an import-competent conformation, or if it also functions as a cytosolic import receptor, actively directing thiolase to the peroxisome.

In contrast, a database search (September, 1999) revealed that malate synthases from most eukaryotic species contain variants of the C-terminal PTS1 motif, while catalases possess either PTS1 variants or are targeted by poorly characterized internal targeting signals (Kragler *et al.*, 1993). The genes for *Y. lipolytica* catalase and malate synthase have not yet been identified, but since targeting signals are evolutionarily conserved, these proteins are unlikely to use the same import receptor as thiolase.

3.13.3 Summary

The results presented identify *Yl*Pex5p as an intraperoxisomal component of the translocation machinery for the majority of peroxisomal matrix proteins, and provide the first direct evidence for the existence of multiple pathways of protein translocation across the peroxisomal membrane.

CHAPTER 4

Functional Analysis of the Domains of the *Yarrowia lipolytica* Peroxin Pex5p

A version of this chapter has been published as "Tetratricopeptide repeat domain of *Yarrowia lipolytica* Pex5p is essential for recognition of the type 1 peroxisomal targeting signal but does not confer full biological activity on Pex5p" (Rachel K. Szilard and Richard A. Rachubinski). Reproduced from ***The Biochemical Journal*, 2000, 346: 177-184** by copyright permission of the Biochemical Society.

4.1 Introductory remarks

As discussed in Chapter 3, the *Y. lipolytica pex5-1* mutant fails to correctly import a subset of peroxisomal matrix proteins, including those with a type 1 peroxisomal targeting signal (PTS1). Pex5 family members have been shown to interact with a PTS1 through their characteristic TPR domain. *In vitro* binding assays were used to investigate the nature of the association of *Yl*Pex5p with the PTS1 signal. A purified recombinant *Yl*Pex5p fusion protein interacted specifically, directly and autonomously with a protein terminating in a PTS1. *In vitro* translated wild-type *Yl*Pex5p specifically recognized functional PTS1s. This activity is abrogated by the substitution of an aspartic acid residue for a conserved glycine residue in the TPR domain (G455D) of the protein encoded by the *pex5-1* allele. Deletion analysis demonstrated that an intact TPR domain of *Yl*Pex5p is necessary, but not sufficient, for both interaction with a PTS1 and functional complementation of a strain lacking *Yl*Pex5p.

4.2 *Yl*Pex5p can specifically recognize a protein terminating in a PTS1

The ability of *Yl*Pex5p to recognize a PTS1 tripeptide was tested using an *in vitro* PTS1 binding assay modelled after that of McCollum *et al.* (1993). GST variants ending in a PTS1 (GST-SKL) or the reverse tripeptide LKS (GST-LKS), which does not resemble a PTS1 but maintains its charge density, were immobilized on glutathione-Sepharose beads. MBP-PEX5, a chimera consisting of *Yl*Pex5p lacking its first 46 amino acid residues fused to the C-terminus of maltose binding protein (MBP), was retained on GST-SKL beads but not on GST-LKS beads (Figure 4-1 A). MBP alone was not retained on either GST-SKL or GST-LKS beads (Figure 4-1 A). Therefore, the MBP-PEX5 fusion protein has specific PTS1

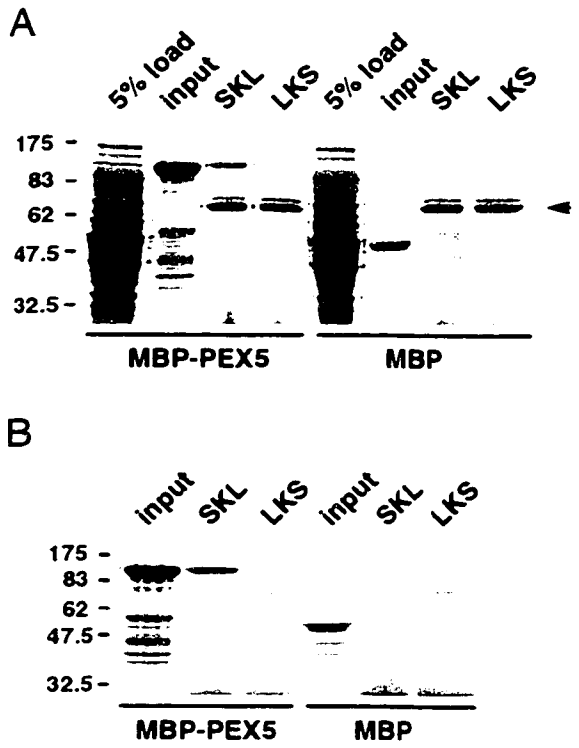


Figure 4-1. A *Yl*Pex5p-MBP chimera recognizes the PTS1 sequence. GST-SKL (*lanes SKL*) and GST-LKS (*lanes LKS*) purified from bacteria were immobilized on glutathione-Sepharose and incubated with bacterially produced, purified MBP-PEX5 protein chimera or an equimolar amount of the control, unfused MBP. (*A*) MBP proteins and immobilized GST proteins were preblocked with buffer containing *E. coli* lysate prior to incubation. The first two wash buffers contained BSA (*arrow*). Bound proteins were eluted by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE and visualized by Coomassie Blue staining. An amount equivalent to 5% of the MBP-PEX5 or MBP preincubation reaction was loaded to show the starting protein profile (*lanes 5% load*). (*B*) MBP proteins and immobilized GST proteins were incubated together without preblocking. No additional proteins were added to the incubation or wash buffers. Bound proteins were eluted and analysed as described above. *Input*, total amount of MBP-PEX5 or unfused MBP used in the binding reaction.

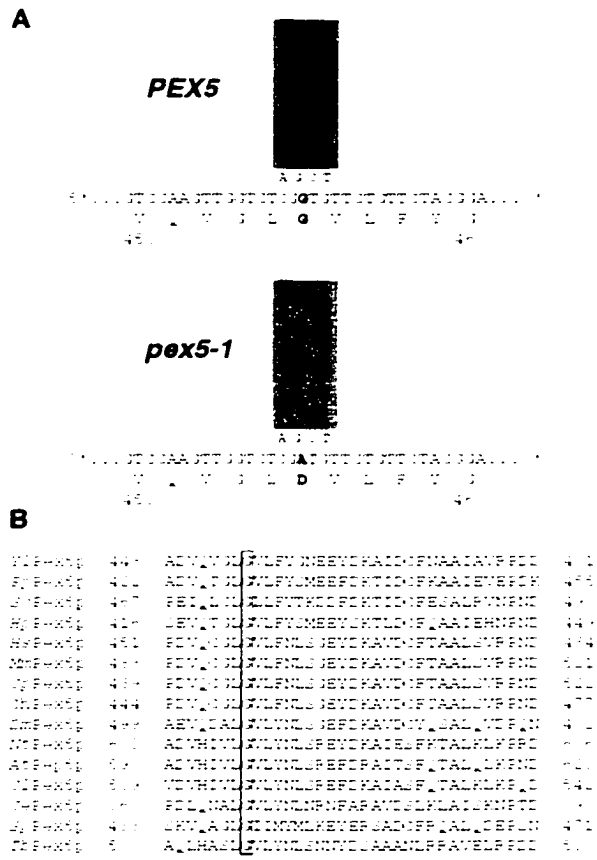
binding activity, which is located in its *Yl*Pex5p domain. The specificity of this interaction is further shown by the fact that the MBP-PEX5 was enriched from a complex mixture of proteins, an *E. coli* lysate, by GST-SKL beads. Binding reactions were repeated with MBP-PEX5 and MBP in the absence of any additional protein during the incubation or wash steps (Figure 4-1 B). MBP-PEX5 was still retained specifically on the GST-SKL beads, suggesting that the interaction between *Yl*Pex5p and PTS1 is direct and does not require other factors.

4.3 The *pex5-1* phenotype arises from the substitution of a conserved amino acid residue in the TPR domain of *Yl*Pex5p

The *pex5-1* strain synthesizes an apparently full-length Pex5 protein that is imported into peroxisomes but fails to associate with anti-SKL-reactive proteins (Chapter 3). The nature of the mutation in *pex5-1* cells was determined by PCR amplification and sequencing of the *PEX5* alleles from both *pex5-1* and wild-type strains. A single change from the wild-type *PEX5* gene was found in the *pex5-1* allele. A transition, G → A, at nucleotide 1364 results in a glycine to aspartic acid substitution at amino acid residue 455 of *Yl*pex5-1p (Figure 4-2 A). Glycine 455 is located in the fifth TPR motif of *Yl*Pex5p, and its functional importance is highlighted by the fact that glycine is conserved at this position in all known Pex5p family members (Figure 4-2 B).

To further demonstrate that the *pex5-1* phenotype was due to the identified mutation, an allele of *PEX5* encoding *Yl*pex5-G455Dp with an aspartic acid residue at position 455 was created by site-directed mutagenesis. The mutant allele was used to replace the wild-type gene in the *E122* strain, generating the strain *pex5-G455D* (Section 2.11.6) (Figure 4-3).

Figure 4-2. Sequence analysis of the *pex5-1* allele. (*A*) Genomic DNA from both the wild-type strain *E122* and the *pex5-1* mutant strain was isolated and amplified by PCR with primers specific for the *PEX5* gene. The amplification products were ligated into plasmid vectors and sequenced. Two independent PCR amplifications were carried out for each template. The results of one amplification are shown for the area of interest, along with the deduced protein sequence from amino acid residues 450-460. The affected base and amino acid residue are indicated in *bold type*. (*B*) Amino acid alignment of the fifth TPR motif of known or putative Pex5p family members. *Numbers* correspond to amino acid residues. The conserved glycine residue corresponding to position 455 of *Yl*Pex5p is *boxed*. *Abbreviations* and accession numbers are as follows: *Yl*, *Yarrowia lipolytica* [SwissProt Q99144]; *Pp*, *Pichia pastoris*, [SwissProt P33292]; *Sc*, *Saccharomyces cerevisiae*, [SwissProt P35056]; *Hp*, *Hansenula polymorpha*, [SwissProt Q01495]; *Hs*, *Homo sapiens*, [SwissProt P50542] (short form); *Mm*, *Mus musculus*, [SwissProt O09012] (long form); *Cp*, *Cavia porcellus* [SwissProt O70525]; Ch, Chinese hamster (*Cricetulus longicaudatus*) [DDBJ AB002564] (short form); *Nt*, *Nicotiana tabacum*, [GenBank AF053104]; *At*, *Arabidopsis thaliana*, [GenBank AF074843]; *Cl*, *Citrullus lanatus*, [GenBank AF068690]; *Dm*, *Drosophila melanogaster* [SwissProt O46085]; *Ce*, *Caenorhabditis elegans* [SwissProt Q18426]; *Sp*, *Schizosaccharomyces pombe* [EMBL AL034342]; *Tb*, *Trypanosoma brucei* [GenBank AF142475].



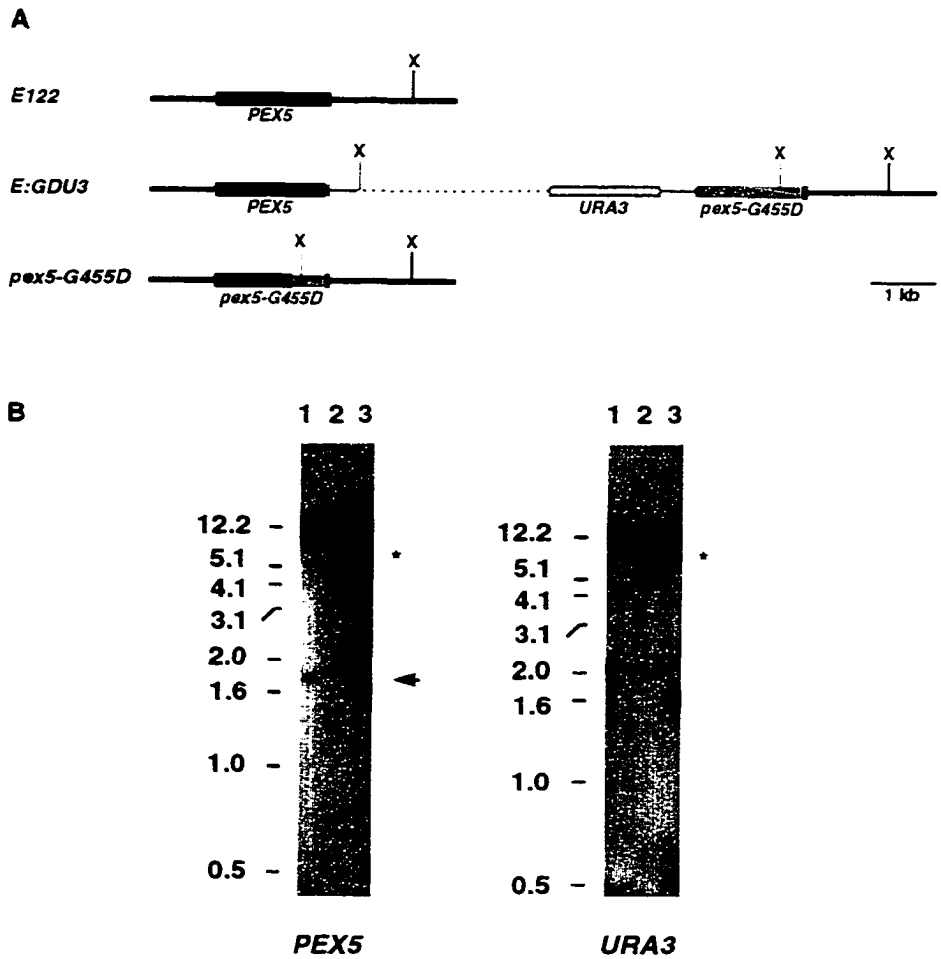


Figure 4-3. Pop in/pop out gene replacement to create the *pex5-G455D* strain. Strains *E:GDU3* and *pex5-G455D* were made as described in Section 2.11.6. (*A*) Restriction endonuclease maps of genomic DNA from wild-type (*E122*) and derivative strains. Regions shown in black are endogenous *Y. lipolytica* DNA. Regions shown in grey are derived from plasmid pGDU3. The dotted region indicates the pBluescriptSKII(-) vector backbone. The open box represents the *URA3* gene. The solid box represents the *PEX5* ORF. Solid lines represent sequences flanking the *PEX5* ORF. The 5' extent of the plasmid-derived region in *pex5-G455D* is drawn arbitrarily. *X*, *Xba*I. (*B*) Southern blot analysis. Genomic DNA from wild-type (*E122*; *lanes 2*), *E:GDU3* (*lanes 3*) and *pex5-G455D* (*lanes 1*) strains was isolated, digested with *Xba*I and processed for Southern blotting as described in Section 2.6.2.1. The probe for the *PEX5* sequence was the *Eco*RI fragment from plasmid p32ΔX (Section 2.11.6). The probe for the *URA3* sequence was the *Sal*I fragment used to create plasmid pGDU3. The *asterisk* indicates the fragment that is diagnostic for the integration of plasmid pGDU3. The *arrowhead* indicates the fragment that is diagnostic for the *pex5-G455D* allele. The *numbers at left* indicate the migration of molecular size markers (in kbp).

The *pex5-G455D* strain manifests the same growth defect on oleate medium as the original mutant *pex5-1* and the *PEX5* gene disruption strain, *pex5-KO* (Figure 4-4). These results confirm that the G455D substitution is sufficient to inactivate *Yl*Pex5p, thus preventing proper peroxisome assembly and function, including the utilization of fatty acids. *Yl*pex5-G455Dp was synthesized at normal levels in the *pex5-G455D* strain (Figure 4-5).

4.4 The G455D mutation abrogates the PTS1 binding activity of *Yl*Pex5p

Full-length wild-type *Yl*Pex5p and *Yl*pex5-G455Dp were tested for their ability to recognize variants of the PTS1 signal. The proteins were translated *in vitro* and labelled with [^{35}S]methionine to facilitate the detection of any weak interactions. Wild-type *Yl*Pex5p was specifically retained on GST-SKL and GST-AKI beads (Figure 4-6, *WT*). The C-terminal tripeptide AKI has been shown to function as a PTS1 in *C. albicans* and *S. cerevisiae* (Aitchison *et al.*, 1991), and *Y. lipolytica* contains peroxisomal proteins that are immunoreactive with anti-AKI antiserum (Aitchison *et al.*, 1992). *Yl*Pex5p was not retained on either GST-LKS beads, where the C-terminal tripeptide does not conform to the PTS1 consensus, or on GST-SKLS beads, where the canonical PTS1 is displaced from the extreme C-terminus by the addition of an extra amino acid residue, a construction that has been shown to prevent the targeting to peroxisomes of a protein normally resident in peroxisomes (Gould *et al.*, 1989). Therefore, wild-type *Yl*Pex5p recognizes only functional PTS1 tripeptides.

In contrast to the wild-type protein, *Yl*pex5-G455Dp was not specifically retained on beads containing any of the PTS1 variants tested (Figure 4-6, *G455D*), suggesting that the glycine to aspartic acid substitution prevents the mutant protein from binding to PTS1s.

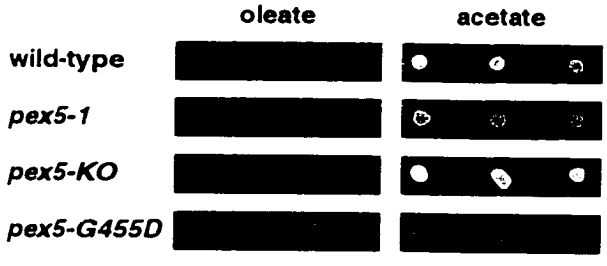


Figure 4-4. Growth of wild-type and *pex5* mutant strains on oleate and acetate. Strains *E122* (*wild-type*), *pex5-1*, *pex5-KO* and *pex5-G455D* were pregrown in YEPD. Cultures were adjusted to the same optical density, and serial 2-fold dilutions were plated on YNO (*oleate*) or YNA (*acetate*). Growth was for 4 d (YNO) or 2 d (YNA) at 30°C.

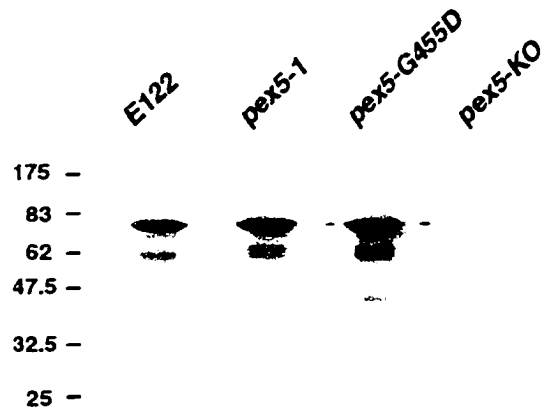


Figure 4-5. Synthesis of Pex5 proteins in wild-type and *pex5* mutant strains. Yeast were pregrown in YEPD and then transferred to YPBO for 6 h. Cell lysates were prepared by disruption with glass beads. 30 μg of each lysate was separated by SDS-PAGE and subjected to immunoblotting with guinea pig anti-*Yl*Pex5p antibodies. The *numbers at left* indicate the migration of molecular size standards (in kD).

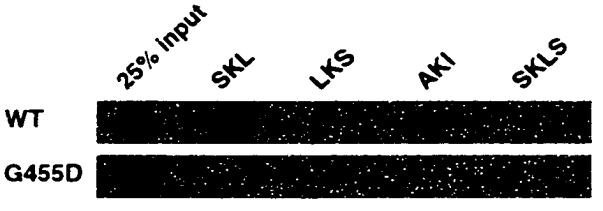


Figure 4-6. Analysis of *Yl*Pex5p and *Yl*pex5-G455Dp PTS1 binding activities. Wild-type *Yl*Pex5p (*WT*) and *Yl*pex5-G455Dp (*G455D*) were labelled with [^{35}S]methionine by *in vitro* transcription/translation. Equimolar amounts of the proteins were preincubated with buffer containing 10 mg BSA/mL prior to incubation with immobilized GST proteins terminating with the sequence SKL, LKS, AKI or SKLS. After extensive washing, bound proteins were eluted by boiling in SDS-PAGE sample buffer and separated by SDS-PAGE. Shown is the fluorograph of the total amount of labelled protein eluted from the GST beads. The signal from an amount equivalent to 25% of the total labelled protein used in the reaction is shown for reference (*25% input*).

These results are in agreement with the previous finding that anti-SKL-reactive proteins associated with *Yl*Pex5p from wild-type cells but not with the mutant form of the protein from *pex5-1* cells (Figure 3-18).

4.5 The TPR domain of *Yl*Pex5p is necessary for its interaction with PTS1 but is not sufficient for functional complementation of a *pex5* deletion mutant

To delineate the region(s) of *Yl*Pex5p involved in PTS1 recognition, truncated variants of *Yl*Pex5p were labelled with [^{35}S]methionine by *in vitro* translation and assayed for their PTS1 binding activity. Deletions of regions N-terminal to the TPR domain did not significantly alter the specific PTS1 binding ability of truncated variants relative to that of wild-type *Yl*Pex5p (Figure 4-7, compare *B* and *C* to *WT*). These results support the observation that in the context of a fusion chimera, the first 46 amino acid residues of *Yl*Pex5p are not required for interaction with a PTS1 (Figure 4-1). In contrast, deletion of the N-terminus and the first half of the TPR domain of *Yl*Pex5p resulted in a variant with no PTS1 binding activity (Figure 4-7, *E*). C-terminally truncated variants that terminate either just before the TPR domain (Figure 4-7, *A*) or two amino acid residues into the fourth TPR motif (Figure 4-7, *D*) failed to show PTS1 binding activity. Removal of the region C-terminal to the TPR domain significantly reduced, but did not completely eliminate, PTS1 binding (Figure 4-7, *F*). This low level of binding was reproducible. Taken together, these results suggest that the N-terminal 300 amino acid residues of *Yl*Pex5p are completely dispensable for PTS1 binding, the C-terminal tail (amino acid residues 550-598) of *Yl*Pex5p is not required for PTS1 recognition but does increase the efficiency of the binding reaction, and the

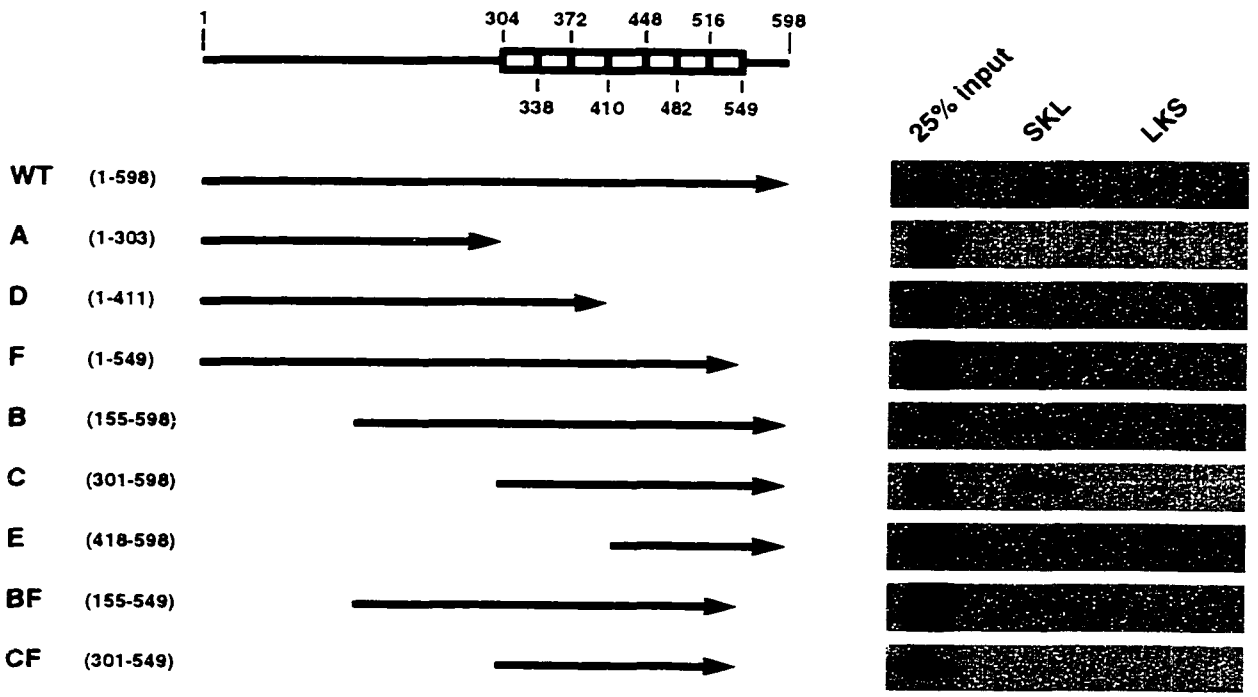


Figure 4-7. Domain analysis of PTS1 binding activity of *Yl*Pex5p. An illustration of *Yl*Pex5p is shown at *top*. The seven TPR motifs are indicated by *boxes*. The *numbers* above and below the illustration refer to the first amino acid residue of the protein or of each TPR motif (1...516), or to the last amino acid residue of the TPR domain or the protein (549, 598). A schematic representation of *Yl*Pex5p and truncation variants used in PTS1 binding assays is shown in the diagram at *left*. The *arrows* indicate the regions present in the wild-type (*WT*) or truncated proteins (*A-CF*). The *numbers in brackets* indicate the amino acid residues present in each protein. There is a non-genomically encoded methionine preceding amino acid residue 418 in mutant E. The results of PTS1 binding assays are presented at *right*. *Yl*Pex5p and variants were labelled with [^{35}S]methionine by *in vitro* transcription/translation. An equimolar amount of each protein was assayed for binding as described in the legend to Figure 4, except in the case of mutant *CF*, where the molar amount of labelled protein (and the total volume of lysate added) was increased four-fold. Overexposures of the *F* and *BF* experiments are shown to permit visualization of weak signals.

TPR domain of *Yl*Pex5p is essential for binding a PTS1. Therefore, the minimum PTS1 binding domain of *Yl*Pex5p was predicted to lie within amino acid residues 301-549. Surprisingly, a protein with these endpoints (Figure 4-7, *CF*) shows no PTS1 binding activity, even when present at four times the molar concentration of wild-type or mutant F proteins. However, a protein consisting of amino acid residues 155-549 of *Yl*Pex5p (Figure 4-7, *BF*) has a low level of PTS1 binding activity similar to that of mutant F, suggesting that the region between residues 155-300 may also have a role in PTS1 recognition and/or in stabilization of the interaction between the targeting signal and its receptor.

The biological activity of *Yl*Pex5p variants was assayed by testing their ability to functionally complement the growth defect of the *pex5-KO* disruption strain on oleate medium (Figure 4-8). The *PEX5* promoter was used to drive the expression of the variant *Yl*Pex5p gene constructs. While all transformants displayed strong growth on medium containing acetate as the carbon source (Figure 4-8, *bottom panel*), which does not require functional peroxisomes for its metabolism, only the transformant carrying a plasmid encoding the wild-type *Yl*Pex5p showed robust growth on oleate medium (Figure 4-8, *middle panel*), which does require functional peroxisomes for its metabolism. The strain synthesizing mutant F, which lacks only the C-terminal end of *Yl*Pex5p, grew slowly on oleate medium. No growth on oleate medium was observed for strains synthesizing any of the other *Yl*Pex5p variants or for the strain carrying the empty vector alone. Cell lysates of the various transformants were analysed by immunoblotting to determine if they were producing the different *Yl*Pex5p variants (Figure 4-8, *top panel*). Appreciable amounts of *Yl*Pex5p variants were synthesized in all cases, except for variant E, where no signal was detected even after

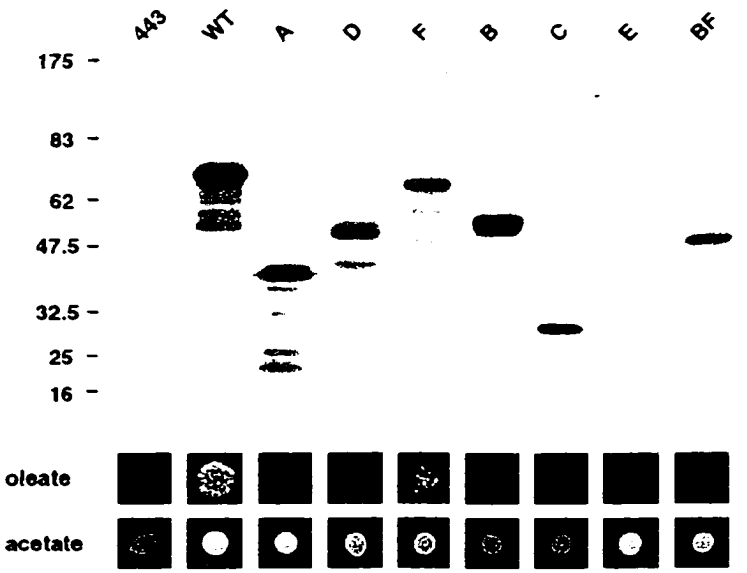


Figure 4-8. Growth characteristics of strains expressing wild-type and truncated forms of *Yl*Pex5p. Lanes are labelled to correspond with the constructs detailed in Figure 5. *Upper panel*, *pex5-KO* cells carrying plasmid pINA443 (*443*) or pINA443 with an insert encoding wild-type (*WT*) or a particular truncated form of *Yl*Pex5p (*A-BF*) were precultured for 16 h in glucose-containing 2xCMD medium and then transferred to oleic acid-containing 2xCMO for 6 h. Cell lysates were prepared by disruption with glass beads. 30 μg of each lysate was separated by SDS-PAGE and subjected to immunoblotting with guinea pig anti-*Yl*Pex5p antibodies. The *upper panel* is a composite of two images; *lanes B* to *BF* were exposed for six times longer than were *lanes 443* to *F*. The *numbers* at *left* indicate molecular size standards (in kD). *Lower panels*, *pex5-KO* strains carrying the plasmids described above were precultured in 2xCMD, adjusted to the same cell density and plated on 2xCMO (*oleate*) or YNA (*acetate*). Growth was at 30°C for 4 d (2xCMO) or 2 d (YNA).

overexposure of the blot (data not shown). From the results of the growth assay (Figure 4-8) and the *in vitro* PTS1 binding data (Figure 4-7), it can be concluded that the ability to recognize a PTS1 is necessary but not sufficient to confer full biological activity on a *Yl*Pex5p variant.

4.6 Discussion

Y. lipolytica and other yeast species have proven to be invaluable tools for the identification of components required for peroxisome assembly (Subramani, 1998; Hettema *et al.*, 1999). The *PEX5* gene of *Y. lipolytica* was identified in a genetic screen for mutants of peroxisome biogenesis, and is very similar in sequence to genes from other organisms that encode proteins that recognize PTS1s (McCollum *et al.*, 1993; Brocard *et al.*, 1994; Dodt *et al.*, 1995; Fransen *et al.*, 1995; Terlecky *et al.*, 1995; Wiemer *et al.*, 1995; Kragler *et al.*, 1998; de Walque *et al.*, 1999).

4.6.1 Substitution of a conserved amino acid residue abrogates the PTS1 binding activity of the Pex5 protein in the pex5-1 strain

While the original mutant strain *pex5-1* synthesizes a Pex5 protein of normal size, unlike the wild-type *Yl*Pex5p, it is not found in association with anti-SKL-reactive proteins *in vivo* (Chapter 3). Analysis of the *pex5-1* allele revealed that the mutant strain produces *Yl*pex5-1p, which contains an aspartic acid substituted for a conserved glycine residue at position 455 (Figure 4-2). The G455D substitution did not support PTS1 binding by the mutant protein in an *in vitro* assay (Figure 4-6), which together with the lack of detectable

PTS1 interaction *in vivo*, suggests a cause for the mutant *pex5-1* phenotype. The data presented here are consistent with a scenario in which the *pex5-1* mutant fails to assemble peroxisomes correctly because of the inability of *Yl*pex5-1p to recognize the PTS1 motif rather than because of its inability to interact with other components of the peroxisomal protein import machinery. The latter possibility could exist, given that the majority of wild-type *Yl*Pex5p is associated with the peroxisomal membrane while peroxisomal *Yl*pex5-1p is found exclusively in the peroxisomal matrix (Figure 3-17). However, any loss of interaction between the mutant Pex5 protein and other components of the peroxisomal translocation machinery would appear to be a secondary effect of the inability of *Yl*pex5-1p to recognize a PTS1, or an additional consequence of a potentially atypical conformation of the mutant protein, since a purified *Yl*Pex5p fusion protein is capable of binding to an SKL tripeptide without the participation of any other factor (Figure 4-1 B).

TPR domains are predicted to form pairs of interlocking antiparallel α-helices with the "knobs" of one helix (formed by bulky residues such as tyrosine or phenylalanine) fitting into the "holes" (formed by small amino acid residues such as glycine or alanine) of the adjacent helix (Goebl and Yanagida, 1991; Lamb *et al.*, 1995; Das *et al.*, 1998). The fact that the mutated amino acid residue in *Yl*pex5-1p is a conserved glycine implies that the structure of the TPR domain may be compromised in the mutant protein. Glycine 455 is the eighth residue of the fifth TPR motif of *Yl*Pex5p. Not only is this glycine conserved in Pex5p family members (Figure 4-2 B), but also, in general, a small amino acid residue is found in the eighth position of repeats of TPR-containing proteins with diverse functions (Goebl and Yanagida, 1991; Lamb *et al.*, 1995; Das *et al.*, 1998). Recently, two inactivating mutations in Chinese

hamster Pex5p were found to involve substitutions of glutamic acid for glycine at amino acid residue 298 (position 7) in the first TPR motif and at amino acid residue 485 (position 8) in the sixth TPR motif (Otera *et al.*, 1998).

4.6.2 The TPR domain of YlPex5p is essential for PTS1 binding

Deletion analysis of *Yl*Pex5p was performed to define the regions needed for PTS1 recognition. The N-terminal 300 amino acid residues of *Yl*Pex5p are not required for its association with a PTS1, because *Yl*Pex5p variants with deletions in this region showed approximately wild-type levels of PTS1 binding activity (Figure 4-7, *WT, B, C*). The TPR domain is essential for the interaction of *Yl*Pex5p with a PTS1, since every truncation tested where part or all of the TPR domain is missing failed to show PTS1 binding activity (Figure 4-7, *A, D, E*). The C-terminal end (residues 550-598) of *Yl*Pex5p is not absolutely required for PTS1 recognition, but its removal significantly reduces the PTS1 binding activity of *Yl*Pex5p (Figure 4-7, *F, BF*). Removal of the C-terminal end of *Yl*Pex5p also makes the region from amino acid residues 155-300 necessary for PTS1 binding (and *vice versa*), since the TPR domain alone does not show PTS1 binding activity (Figure 4-7, *CF*). An intact TPR domain has been shown to be required for full PTS1 binding activity of *S. cerevisiae* Pex5p (Brocard *et al.*, 1994). There appear to be residues critical for PTS1 binding outside the TPR motifs of *Sc*Pex5p, since deletion of the C-terminal 48 amino acid residues abolishes PTS1 binding, and a *Sc*Pex5p variant that initiates 6 amino acid residues before the TPR domain has only very weak PTS1 binding activity. In contrast, the first three TPR motifs of *P. pastoris* Pex5p are sufficient, and necessary, for binding a PTS1 *in vitro* (Terlecky *et al.*, 1995).

Truncation mutant D of *Yl*Pex5p (Figure 4-7) was designed to mimic the C-terminal deletion in the minimal PTS1 binding domain of the *Pp*Pex5p (*i.e.*, termination after the second amino acid residue of the fourth TPR motif), but no interaction was detected between mutant D and GST-SKL. Therefore, it appears that in terms of the structural requirements for PTS1 binding, *Yl*Pex5p is more similar to *Sc*Pex5p than it is to *Pp*Pex5p.

4.6.3 The PTS1 binding domain of YlPex5p is not sufficient for complete biological function

The role of *Yl*Pex5p in peroxisome biogenesis is greater than simply its ability to bind a PTS1 tripeptide, since the extent of PTS1 binding by a *Yl*Pex5p variant (Figure 4-7) did not necessarily predict its ability to restore peroxisome function in a *pex5* gene disruption strain (Figure 4-8). Variants B and C (which lack the N-terminal 154 and 300 amino acid residues of Pex5p, respectively) showed approximately wild-type levels of PTS1 binding activity, but were unable to complement the *pex5-KO* strain for growth on oleic acid media. Mutant F, which lacks the C-terminal 48 amino acid residues of *Yl*Pex5p, showed weak PTS1 binding and was able to partially complement the *pex5-KO* strain. Mutant BF (residues 155-549) also bound GST-SKL weakly but did not complement the *pex5-KO* strain, reinforcing the functional importance of the N-terminal region of *Yl*Pex5p.

Several reasons can be put forward to explain why a mutant *Yl*Pex5p that retains PTS1 binding ability might not be fully biologically functional: 1) The deleted region of *Yl*Pex5p could include information required to direct it to peroxisomes. *Yl*Pex5p does not have a recognizable PTS, and the domain responsible for its peroxisomal targeting has yet to

be characterized; 2) The mutant *Yl*Pex5p might fail to interact with other components of the peroxisomal translocation machinery. Peroxin binding partners for *Yl*Pex5p await identification; 3) The mutant *Yl*Pex5p might not assume the correct quaternary protein structure. Purified human Pex5p produced in *E. coli* has recently been shown to exist as a homotetramer, and the domain responsible for oligomerization was located within the N-terminal 213 amino acid residues (Schliebs *et al.*, 1999); 4) Truncated forms of *Yl*Pex5p may lack a domain or domains needed for the release of PTS1-bearing proteins in a timely fashion, resulting in prolonged lives for complexes of *Yl*Pex5p and cargo proteins and preventing *Yl*Pex5p from recycling to pick up new cargo.

4.6.4 Summary

The results presented demonstrate that *Yl*Pex5p can bind specifically, directly and autonomously with functional PTS1 motifs. An intact TPR domain of *Yl*Pex5p is necessary, but not sufficient, for both interaction with a PTS1 and functional complementation of a strain lacking *Yl*Pex5p.

CHAPTER 5

Perspectives

5.1 Synopsis

The *pex5-1* strain is one of a series of *pex* mutants of *Y. lipolytica* that was generated with the aim of achieving a greater understanding of the molecular mechanisms involved in peroxisome biogenesis. Cloning of the *PEX5* gene and analysis of its encoded peroxin strongly suggest that *Yl*Pex5p is an intraperoxisomal PTS1-binding protein that is essential for the translocation of most peroxisomal matrix proteins.

5.2 Future directions for research

To date, no protein has been demonstrated to make up part of the putative protein conducting channel of the peroxisomal membrane. The fact that peroxisomal p62 is tightly associated with the peroxisomal membrane in *pex5* mutant strains offers a possible method of identifying channel components, as well as other elements of the translocation apparatus. The membrane fraction of peroxisomes isolated from a *pex5* mutant strain could be exposed to cross-linking agents and then solubilized with detergent. Subsequent immunoprecipitation with anti-SKL antibodies would be expected to co-precipitate proteins in close proximity to p62. Treating the membranes with different stripping agents (*e.g.* NaCl, urea, carbonate) prior to cross-linking and comparison of the co-immunoprecipitated polypeptides might identify distinct p62-containing complexes, possibly reflecting different stages of import such as docking, insertion and translocation.

The main feature of *Yl*Pex5p that distinguishes it from other Pex5 family members is that the *Y. lipolytica* protein is detected exclusively in the peroxisomal matrix. A number of questions arise from this observation. What is the signal that directs *Yl*Pex5p to the

peroxisomal matrix? This protein contains neither a PTS1 nor a PTS2. Do some species possess a mechanism that actively promotes retrograde transport of empty receptors from the peroxisomal matrix back to the cytosol, a mechanism that is absent from *Y. lipolytica* peroxisomes? Or conversely, if some putative *Y. lipolytica* import receptor(s) is able to be translocated in both directions across the peroxisomal membrane, does *Yl*Pex5p contain a peroxisome retention signal that keeps it in the peroxisomal matrix? This scenario seems less likely, since there is no evidence for a significant efflux of proteins from the peroxisomal matrix. Determination of the subcellular localization of the *Yl*Pex5p truncation mutants synthesized *in vivo* should be useful in addressing some of these issues.

The strongest evidence that the human Pex5p is a shuttling receptor is that the location of the receptor can be altered by changing cellular conditions. For example, inhibition of translocation by depletion of ATP or by incubating cells at 16°C resulted in a shift in *Hs*Pex5p distribution from predominantly cytosolic, to mostly peroxisomal. Shifting cells from 16°C to 37°C caused *Hs*Pex5p to reassume a primarily cytosolic localization; a further return to translocation-inhibiting conditions once again caused an accumulation of *Hs*Pex5p on peroxisomes (Dodt and Gould, 1996). If cellular conditions were to be identified that specifically altered *Yl*Pex5p distribution, these conditions might be used to elucidate distinct steps in the *Yl*Pex5p-dependent import pathway.

It will also be important to identify other peroxins with which *Yl*Pex5p interacts, and to determine other functions (if any) of the domains of this protein. One role of the TPR domain of *Yl*Pex5p is PTS1 recognition, but this region may have other yet undefined functions. The TPR domain of *Hs*Pex5p interacts with the zinc-binding domain of *Hs*Pex12p

(Chang *et al.*, 1999). The region of Pex5p that associates with Pex13p has not yet been rigorously defined, but the first 43 amino acid residues of Pex5p from *S. cerevisiae* are not necessary for interaction with *Sc*Pex13p in a two-hybrid assay (Elgersma *et al.*, 1996a). The N-terminus of *Hs*Pex5p has been implicated in both homotetramer formation and interaction with Pex14p (Schliebs *et al.*, 1999). However, the Pex5p-interaction domains of the PMPs Pex12p, Pex13p and Pex14p are on regions of these proteins that are believed to face the cytosol, thus making it unlikely that putative *Y. lipolytica* orthologues of Pex12p, -13p and -14p could interact with intraperoxisomal *Yl*Pex5p. The function of the N-terminus of *Yl*Pex5p is as yet unknown. The oligomerization state of *Yl*Pex5p has not yet been studied. However, if *Yl*Pex5p also forms a homotetramer and if this configuration requires the presence of the N-terminus, then it could be concluded that oligomerization of *Yl*Pex5p is not required for PTS1 binding, since deletion of regions N-terminal to the TPR domain do not affect recognition of an SKL tripeptide. It will also be interesting to determine if the soluble pool of *Yl*Pex5p interacts with any proteins besides the anti-SKL-reactive proteins p62 and p64, and if the "free" *Yl*Pex5p (*i.e.* not complexed with p62/p64) is actually in association with some other import substrate(s) that is not recognized by anti-SKL antibodies, or possibly with regulatory factors that modulate the PTS1 binding or membrane-association function of *Yl*Pex5p.

5.3 Pex5 proteins appear to have distinct functions in different organisms

A comparison of the proposed mechanisms of peroxisomal matrix protein import in different systems, highlighting the roles of Pex5 proteins, is shown in Figure 5-1. Panel A

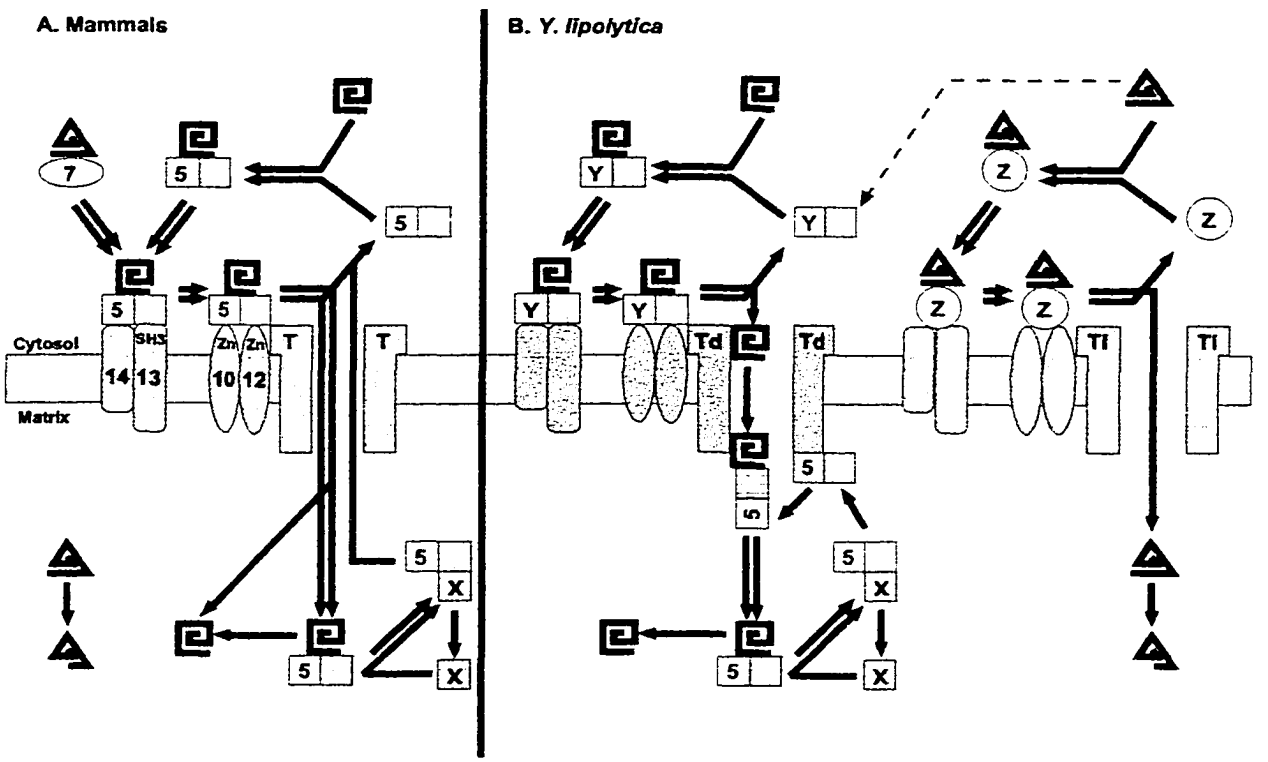


Figure 5-1. Peroxisomal matrix protein import in different systems. (*A*) Conventional model for the import of matrix proteins into peroxisomes, primarily based on studies of mammalian peroxins. (*B*) Model for the import of matrix proteins into *Y. lipolytica* peroxisomes. See text for details. For simplicity, all proteins are drawn as monomers, regardless of their true quaternary structure. *Square coils*, PTS1-containing proteins. *Triangular coils*, PTS2-containing proteins. *Numbers* indicate characterized peroxins. *X*, putative Pex5p regulatory factor. *Y*, putative cytosolic PTS1 receptor. *Z*, putative cytosolic receptor(s) for proteins imported primarily through the *Yl*Pex5p-independent pathway. *T*, translocon. *Td*, *Yl*Pex5p-dependent translocon. *Ti*, *Yl*Pex5p-independent translocon. *SH3*, SH3 domain. *Zn*, zinc binding domain. *Hatched areas*, known or potential TPR domains.

illustrates what could be considered the "conventional" view of Pex5p-mediated import. This model is primarily based on experiments investigating Pex5p and associated factors in humans and other mammals (Dodt *et al.*, 1995; Fransen *et al.*, 1995; Wiemer *et al.*, 1995; Gould *et al.*, 1996; Dodt and Gould, 1996; Otera *et al.*, 1998; Braverman *et al.*, 1998; Fransen *et al.*, 1998; Schliebs *et al.*, 1999; Will *et al.*, 1999; Shimizu *et al.*, 1999; Chang *et al.*, 1999). When considering other systems (not including *Y. lipolytica*), some differences may exist, but overall there seems to be more agreement than disagreement. Pex5p and newly synthesized proteins destined for the peroxisomal matrix are initially free in the cytosol, where the C-terminal TPR domain of Pex5p binds to the PTS1 of the future peroxisomal matrix protein. The loaded receptor then moves to the membrane and associates with the Pex13p-Pex14p docking complex. Pex7p, loaded with a PTS2-containing protein, uses the same docking complex, and (in mammals) may require the long form of Pex5p to facilitate docking. Next, the Pex10p-Pex12p complex accepts the loaded PTS1 receptor and transfers it to the putative translocation channel (PexTp). The PTS1-containing protein then moves across the peroxisomal membrane, either with or without Pex5p. If the PTS1-containing protein is translocated by itself, empty Pex5p is released directly to the cytosol from the outer surface of the peroxisomal membrane. If Pex5p has been co-imported with the PTS1-containing protein, the participation of another protein (PexXp) may be required to facilitate the release of the PTS1-containing protein. The unloaded Pex5p is then exported back across the peroxisomal membrane to the cytoplasm for another round of import. In this illustration, Pex5p is shown entering and exiting the peroxisome via the same translocon, however it is also possible that a distinct translocon capable of retrograde protein trafficking may exist.

The sequence of events for Pex7p-dependent import of PTS2-containing proteins is not shown in Figure 5-1 A, but may be the same as that shown for Pex5p-dependent import of PTS1-containing proteins.

The left side of Figure 5-1 B illustrates a model for the import of PTS1-containing proteins into *Y. lipolytica* peroxisomes. Events in the cytosol and at the outer face of the peroxisomal membrane are illustrated as being essentially the same as those in the conventional model, although none of the responsible proteins has been identified at this time. PexYp represents a putative receptor for PTS1-containing proteins in the cytosol. PexYp may or may not be a TPR-containing protein. PexYp is shown here as a receptor that cycles between the cytosol and the peroxisomal membrane. However, it could equally be permanently membrane-bound, diffusing laterally through the membrane to interact with the putative docking complex and translocon. The PTS1-containing protein disengages from PexYp and associates tightly with the putative *Yl*Pex5p-dependent translocation channel (PexTdp), with its C-terminus shielded from the cytosol. Next, intraperoxisomal *Yl*Pex5p binds to the C-terminal PTS1 of the partially translocated protein, pulling it completely through the translocation channel and eventually into the matrix. The PTS1-containing protein is then released from *Yl*Pex5p, possibly with the assistance of a *Yl*Pex5p-regulatory factor (PexXp). The unloaded *Yl*Pex5p then recycles back to the membrane, ready to pull another PTS1-containing protein through the translocation channel. Therefore, this model predicts that *Yl*Pex5p is a cycling receptor, but that its cycle is restricted to the peroxisomal matrix and the inner face of the peroxisomal membrane. According to this model, in the absence of *Yl*Pex5p function, the import of most proteins is blocked at the level of tight

association with the translocation channel (*i.e.* carbonate-inextractable proteins specifically found in the peroxisomal membrane fractions from *pex5* mutant strains). The translocation blockage also causes an accumulation of peroxisomal proteins at the outer surface of the peroxisomal membrane (*i.e.* carbonate-extractable proteins specifically found in the peroxisomal membrane fractions from *pex5* mutant strains), and reduces the efficiency of targeting of matrix proteins (*i.e.* proteins mislocalized to the cytosol in *pex5* mutant strains).

The right side of panel B illustrates the import of PTS2-containing proteins (such as thiolase) into *Y. lipolytica* peroxisomes, but it could also represent an analogous import pathway (or pathways) for proteins with other uncharacterized PTSs (such as malate synthase and catalase) that are successfully translocated into peroxisomes in *pex5* mutant strains. These proteins are imported into peroxisomes primarily via distinct receptor(s) (represented by PexZp), docking complex(es) and *Yl*Pex5p-independent translocon(s) (PexTip). There is currently no evidence to suggest that thiolase and other proteins that do not depend on *Yl*Pex5p for import are co-imported into peroxisomes along with their receptors. The dashed arrow indicates that these proteins may use the *Yl*Pex5p-dependent import pathway to a minor extent.

While the search for *PEX* genes in *Y. lipolytica* has by no means been exhausted, especially considering that to date only one selection strategy has been used, it is nonetheless curious that there has not yet been a *Y. lipolytica* peroxin identified that is an orthologue of any of the peroxins that have been shown to interact with (either directly or indirectly), and/or specifically affect the localization of, Pex5p from other organisms (Pex4p (van der Klei *et al.*, 1998), Pex7p (Rehling *et al.*, 1996; Girzalsky *et al.*, 1999), Pex10p (Dodt and Gould, 1996;

Chang *et al.*, 1999), Pex12p (Dodt and Gould, 1996; Chang *et al.*, 1999), Pex13p (Gould *et al.*, 1996; Elgersma *et al.*, 1996a; Erdmann and Blobel, 1996; Girzalsky *et al.*, 1999), Pex14p (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Fransen *et al.*, 1998; Will *et al.*, 1999; Girzalsky *et al.*, 1999; Shimizu *et al.*, 1999), Pex17p (Huhse *et al.*, 1998)). This may further support the contention that the cellular function of *Yl*Pex5p is fundamentally different than that of other Pex5 family members, and may suggest that not all mechanisms of peroxisome biogenesis are evolutionarily conserved, even one so essential as matrix protein import. If the validity of this supposition is borne out by experimental evidence, it would be advisable to consider reassigning *Yl*Pex5p to an independent peroxin group, or at least designating it as a distinct isoform (*e.g.* Pex5βp). Only the continued identification of novel *Y. lipolytica* peroxins and the elucidation of their coordinated mechanisms of action will reveal if this hypothesis is correct.

CHAPTER 6

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APPENDIX 1

Alignment of *Yl*Pex5p with some representative members of the Pex5 family

*Yl*Pex5p and known or predicted Pex5 proteins (one from each of the major types of organisms, if there are multiple examples) were aligned using the CLUSTAL multiple sequence alignment program in the PC-GENE software package (IntelliGenetics). Abbreviations: YL, *Yarrowia lipolytica*; PP, *Pichia pastoris*; HS, *Homo sapiens* (short form); DM, *Drosophila melanogaster*; TB, *Trypanosoma brucei*; NT, *Nicotiana tabacum*; CE, *Caenorhabditis elegans*. For accession numbers, refer to the legend to Figure 4-2. A star indicates that a position in the alignment is perfectly conserved. A dot indicates that a position is well conserved.

```
YLPEX5P     MSFMR-GGSE--CSTGRN-------------------------------    15
PPPEX5P     MSLIG-GGSD--CAAGSN-------------------------------    15
HSPEX5S     MAMRELVEAE--CG-GAN-------------------------------    15
DMPEX5P     MSFRPLVEGD--CG-GVN-------------------------------    15
TBPEX5P     MD----------CGAGFALGQQLAKDALHMQGGVR---------PGTTGN    31
NTPEX5P     MAMRDLVTGAPSCGEPSSSSNPLGALANALIGSSSKTQERLKEIPTSVST    50
CEPEX5P     M--KGVVEGQ--CGQ----------------------QNALVGLANTFGT    24
            *           *.

YLPEX5P     ------------PLSQF--------------TKHTAEDRSLQHD-RVAGP    38
PPPEX5P     ------------PLAQF--------------TKHTQHDTSLQQS-MRNGE    38
HSPEX5S     ------------PLMKL--------------AGHFTQDKALRQEGLRPGP    39
DMPEX5P     ------------PLMQL--------------GGQFTRDVAHKDEGYVQRH    39
TBPEX5P     VEQDALMTGMMVP----PTGPMED----------WAQHFAAHQHHHQ---    64
NTPEX5P     SSDGNFLAGFQEPLVSLPGSEFEQPLHPNIQGSQFLQGFRSADQNRLSDA   100
CEPEX5P     SNQRVAPSNAAASL--LPSSSMGE-------------------------    46
                      .

YLPEX5P     SGGRVGGM----RSNTGEMS-QQDR----------EMMARFGAA------    67
PPPEX5P     FQQGNQRM----MRNESTMS-PMER----------QQMDQF-MQ------    66
HSPEX5S     WPPGAPAS----EAASKPLGVASED----------ELVAEFLQD------    69
DMPEX5P     F----------ERAARP-----ED----------QLINEFLGQ------    57
TBPEX5P     ----QHQQMMMQRQHNDALMIQQQHRDME------------EAFRASAR    97
NTPEX5P     WDEIQRPQLPFGSQNMTNIPLEHARVQPDLNGPPQQVLSSFLHSFVNSSQ   150
CEPEX5P     ----------------------------------QMANEFL--------    53

YLPEX5P     -------G------PEQ----------------------SSFNYEQMRH-    81
PPPEX5P     -------Q------QNN----------------------PAFNFQPMQH-    80
HSPEX5S     -------QNAPLVSRAP----------------------QTFKMDDLLA-    89
DMPEX5P     -------VTAP-----P----------------------QSFQMDTLLQ-    72
TBPEX5P     AGAPQQANAGPLM-MPPGPMMMAGGMAPMM-------HAGGFMMGGMPQM   139
NTPEX5P     GGMQFRPTSLPLLGLSEGDKQCIRDRSTIMARHFFADKGEDFINGQVNAL   200
CEPEX5P     ----------------------RQQARTMAP-------TSFSMKSMQNN    73
                                                  .*     .

YLPEX5P     ------ELHNMGAQG--GQIPQVPS--------QQGAANGGQWARDF---   112
PPPEX5P     ------ELNVMQQNM--NAPQQVAN--------NS-------WNQEFRMK   107
HSPEX5S     ------EMQQIEQSNFRQAPQRAPG--------VADLALSENWAQEF---   122
DMPEX5P     ------EMRDI---NIHGNPQQQMH--------SQQ---AEQWGQDFARG   102
TBPEX5P     MPCAPMGMN-MGMAPVATMSPATTN------TVSGAREGATA-VSSAAPG   181
NTPEX5P     LSSLEIDNDARARGPVPGRYPELEEYWNESLAMRPAPHVADGWINEFAQN   250
CEPEX5P     LP-----------------------------QASASSSLAANWTKEFQPR    94
                                                        .

YLPEX5P     ----GGQQT--APGAAPQDAKNWNAEFQRGGSPAEAMQQQGPGPMQGGMG   156
PPPEX5P     DPMVANAPS--AQVQTPVQSTNWAQDFQQAGPEVQHHAQQHQHPILSVPG   155
HSPEX5S     LAAG---DA--VDVTQDYNETDWSQEF----------ISEVTDPLSVSPA   157
DMPEX5P     LAPALPNKM--IHMQAQQQDLQHAQEF----------FDE---PLISSQN   137
TBPEX5P     VVDLGGDSAWAEKLHQAE----WGQDY------------KDVEVHTVEG   214
NTPEX5P     RVEHADPNAWAQSFEQQHGANGWASEFEHEQSQLGMIGQMRGANIPNLAA   300
CEPEX5P     --------------QNQLASQWSQQYTSSAPSMESAWRQVQAPSMTSTS   129
                           . ..                          .
```

```
YLPEX5P     MGGMPMYGMA--RPMYSGMSANMAPQFQPQQANARVVELDEQNWEEQFKQ   204
PPPEX5P     VRA-GIYGGG--RLMGGSMMNRAAQMQQQNPAQAQTSEQSQTQWEDQFKD   202
HSPEX5S     RWAEEYLEQSEEKLWLGEPEGTATDRWYDEYHPEEDLQHTASDFVAKVDD   207
DMPEX5P     FRSLPPLRQP----LMPIAAGQQQDPFFDS-----AMETIITDHLPQAPQ   178
TBPEX5P     STAQTVEEHA---------KTSKFYEFMDKIRKKELLVDEDSGEVVQGPG   255
NTPEX5P     MEQTRMLAHTLAQNNDPKFQNSKFLQFVSKMSRGEITIEENQFKPATVAP   350
CEPEX5P     --------------------------------------------------   129

YLPEX5P     MDSAVGKGKE---VEEQTAETATATETVTETETTTEDKPMDIKNMDFENI   251
PPPEX5P     IESMLNS-----------------------KTQEPKTKQQEQNTFEQV    227
HSPEX5S     -PKLANSEGT---SDAWVDQFTRPVNTSALDMEFERAKSAIESDVDF---   250
DMPEX5P     -------GES---LDDWISDYQRSTE--------QKEQTAANFNEKF---   207
TBPEX5P     PDPDVEADTEYLA------RLAAMEGINVPPSVMDHMQGQDGVQRGTDED   299
NTPEX5P     GDWAAEYGQQYNGGQSWADQFAHEELSRGPQGWVNEFSAEREQHGSVNDE   400
CEPEX5P     ------SHQPITDAGMWSSEYLDTVDTSLTKSSGT------------QN   160

YLPEX5P     W----KNLQVN-VLDNMDEWLEET------NSPAWERDFHEYTHNRPEFA   290
PPPEX5P     W----DDIQVS-YADV--ELTNDQ------FQAQWEKDFAQYAEGRLNYG   264
HSPEX5S     W----DKLQAE-LEEMAK---RDA------EAHPWLSDYDDLTSATYD-K   285
DMPEX5P     W----ERLQDE-WQKLA-----DE------NEHPWLSEYND-NMDAY--K   238
TBPEX5P     ---------------MEGMMGDDVYDPSADVEQWAQEYAQMQAMQERLQ    333
NTPEX5P     WVNEFSKLNVNDWADEFGNQVAEGAFG-ETSADSWAEAYDEYMNEQAALK   449
CEPEX5P     WADDF--MEQQDNYGMENTWKDAQAFE-----QRWEEIKRDME------K   197
                                    .          *     .

YLPEX5P     D--------YQFEENNQFMEHPDPFKIGVELMETGGRLSEAALAFEAAVQ   332
PPPEX5P     E--------YKYEEKNQFRNDPDAYEIGMRLMESGAKLSEAGLAFEAAVQ   306
HSPEX5S     G--------YQFEEENPLRDHPQPFEEGLRRLQEGD-LPNAVLLFEAAVQ   326
DMPEX5P     E--------YEFAEGNPMSDVENPFEKGKEYLSKGD-IPSAVLCFEVAAK   279
TBPEX5P     NNTD-----YPFEANNPYMYHENPMEEGLSMLKLAN-LAEAALAFEAVCQ   377
NTPEX5P     QQSDASRGVYVFSDLNPYVGHPNPLKEGQELFRKGL-LSEAVLALEAEVL   498
CEPEX5P     DESLQSPENYVYQEANPFTTMSDPLMEGDNLMRNGD-IGNAMLAYEAAVQ   246
                      * . . *.      ..   *   .  .  .  .* *  *.

YLPEX5P     KNTEHAEAWGRLGACQAQNEKEDPAIRALERCIKLEPGNLSALMNLSVSY   382
PPPEX5P     QDPKHVDAWLKLGEVQTQNEKESDGIAALEKCLELDPTNLAALMTLAISY   356
HSPEX5S     QDPKHMEAWQYLGTTQAENEQELLAISALRRCLELKPDNQTALMALAVSF   376
DMPEX5P     KQPERAEVWQLLGTSQTENEMDPQAIAALKRAYDLQPDNQQVLMALAACY   329
TBPEX5P     KEPEREEAWRSLGLTQAENEKDGLAIIALNHARMLDPKDIAVHAALAVSH   427
NTPEX5P     KNPENAEGWRLLGIAHAENDDDQQAIAAMMRAQEADPTNLEVLLSLGVSH   548
CEPEX5P     KDPQDARAWCKLGLAHAENEKDQLAMQAFQKCLQIDAGNKEALLGLSVSQ   296
            ....    *  **  ...*. .  .. *. .    .. .  .  .*. .

YLPEX5P     TNEGYENAAYATLERWL--ATKYPEVVDQARN---QEPRLGNEDKF----   423
PPPEX5P     INDGYDNAAYATLERWI--ETKYPDIASRARS---SNPDLDGGDRI----   397
HSPEX5S     TNESLQRQACETLRDWLRYTPAYAHLVTPAEE-GAGGAGLGPSKRILGSL   425
DMPEX5P     TNEGLQNNAVRMLCNWLTVHPKYQHLVAAHPELQAEGTSLA------SSL   373
TBPEX5P     TNEHNANAALASLRAWLLSQPQYEQLGSVNLQADVDIDDLNVQSEDF-FF   476
NTPEX5P     TNELEQQAALKYLYSWLRHHPKYGSIAPQD-----------QPVSF-YH   585
CEPEX5P     ANEGMENEALHQLDKWM---SSY---------LGSNSTQVTTTPPLYSSF   334
             *.  ...*   *  *.   . *
```

```
YLPEX5P     ---QLHSRVTELFIRAAQLSPDGANIDADVQVGLGVLFYGNEEYDKAIDC   470
PPPEX5P     ---EQNKRVTELFMKAAQLSPDVASMDADVQTGLGVLFYSMEEFDKTIDC   444
HSPEX5S     LSDSLFLEVKELFLAAVRLDP--TSIDPDVQCGLGVLFNLSGEYDKAVDC   473
DMPEX5P     IGPSKLRDLQQIYLEAVRQHP--SEVDAEVQDALGVLYNLSGEFDKAVDC   421
TBPEX5P     AAPNEYRECRTLLHAALEMNPN----DAQLHASLGVLYNLSNNYDSAAAN   522
NTPEX5P     A------DVSRLFTDAAQMSPD----DADVHIVLGVLYNLSREYDKAIES   625
CEPEX5P     LDSDTFNRVEARFLDAAR--QQGATPDPDLQNALGVLYNLNRNFARAVDS   382
                          *    .    *.... ****.    ..... .

YLPEX5P     FNAAIAVRPDDALLWNRLGATLANSHRSEEAIDAYYKALELRPSFVRARY   520
PPPEX5P     FKAAIEVEPDKALNWNRLGAALANYNKPEEAVEAYSRALQLNPNFVRARY   494
HSPEX5S     FTAALSVRPNDYLLWNKLGATLANGNQSEEAVAAYRRALELQPGYIRSRY   523
DMPEX5P     YQSALQVDPQNAKTWNRLGASLANGSRSVEAVEAYQQALQLQPGFIRVRY   471
TBPEX5P     LRRAVELRPDDAQLWNKLGATLANGNRPQEALDAYNRALDINPGYVRVMY   572
NTPEX5P     FKTALKLKPRDYSLWNKLGATQANSVQSADAILAYQQALDLKPNYVRAWA   675
CEPEX5P     LKLAISKNPTDARLWNRLGATLANGDHTAEAISAYREALKLYPTYVRARY   432
               *.   * .   **.***. **  .. .*. **  **.. *...*

YLPEX5P     NLGVSCINIGCYKEAAQYLLGALSMHK----VEGVQDDVLAN-------Q   559
PPPEX5P     NLGVSFINMGRYKEAVEHLLTGISLHE----VEGVDASEMSS---NQGLQ   537
HSPEX5S     NLGISCINLGAHREAVEHFLEALNMQRKSRGPRG-----------EGGAM   562
DMPEX5P     NVGVCCMNLKAYKEAVEHLLTALTMQAHTNAARELPNAAMAATFRGQNQM   521
TBPEX5P     NMAVSYSNMSQYDLAAKQLVRAIYMQVGGTTPTGEASRE----------A   612
NTPEX5P     NMGISYANQGMYEDSIRYYVRALAMN-----PKADNA-------------   707
CEPEX5P     NLGISCMQLSSYDEALKHFLSALELQKGGNDASGIWTTMRSAAIRTSNVP   482
            *....  .   .  .    . .. ..       .

YLPEX5P     STNLYDTLKRVFLGMDRRDLVAKVGNGMDVNQFRNEFEF-----   598
PPPEX5P     NNALVETLKRAFLGMNRRDLVDKVYPGMGLAQFRKMFDF-----   576
HSPEX5S     SENIWSTLRLALSMLGQSDAYGAA-DARDLSTLLTMFGLPQ---   602
DMPEX5P     SESIWSTLKMVISLMGRSDLQSYV-SDRNLAALNEAF--KD---   559
TBPEX5P     TRSMWDFFRMLLNVMNRPDLVELTYA-QNVEPFAKEFGLQSMLL   655
NTPEX5P     ----WQYLRISLSCASRNDMLEACDA-RNLDVLQKEFPL-----   741
CEPEX5P     DNLLRA--------VERRDLAA----------------VKASLV   502
                         .. *  .
```

```
Consensus length:  794
Identity  : 45 ( 5.7%)
Similarity: 88 ( 11.1%)
```