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**PAS7 ENCODES A NOVEL YEAST MEMBER OF THE WD-40 PROTEIN FAMILY
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PAS7 encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes

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To identify components of the peroxisomal import pathway in yeast, we have isolated *pas* mutants affected in peroxisome biogenesis. Two mutants assigned to complementation group 7 define a new gene, *PAS7*, whose product is necessary for import of thiolase, a PTS2-containing protein, but not for that of SKL (PTS1)-containing proteins, into peroxisomes. We have cloned *PAS7* by complementation of the oleic acid non-utilizing phenotype of the *pas7-1* strain. The DNA sequence predicts a 42.3 kDa polypeptide of 375 amino acids encoding a novel member of the β -transducin related (WD-40) protein family. A Myc epitope-tagged Pas7p, expressed under the control of the *CUP1* promoter, was functionally active. Subcellular localization studies revealed that in the presence of thiolase this epitope-tagged Pas7p in part associates with peroxisomes. However, in a thiolase-deficient mutant, Pas7p was entirely found in the cytoplasm. We suggest that Pas7p mediates the binding of thiolase to these organelles.

Key words: 3-oxoacyl-CoA thiolase/peroxisomal targeting signal/peroxisome/protein sorting/*Saccharomyces cerevisiae*

Introduction

The ability to direct proteins accurately and efficiently to different compartments is a fundamental feature of eukaryotic cells and requires an elaborate delivery system. Protein sorting is mediated by targeting signals contained within concise amino acid sequences and the recognition of such signals by specific signal sequence receptors which direct proteins to their destination.

Signal sequences that target proteins to the ER, mitochondria or chloroplasts are generally found in amino-terminal prepieces which are cleaved upon entry of the proteins into the organelles (Gierasch, 1989; von Heijne, 1990). In contrast, most peroxisomal proteins are not proteolytically processed during import. Many peroxisomal proteins contain a carboxy-terminal Ser-Lys-Leu (SKL) tripeptide peroxisomal targeting signal (PTS1; for a review see De Hoop and Ab, 1992; Subramani,

1993). PTS1 was initially identified in firefly luciferase (Gould *et al.*, 1987) and is sufficient to target proteins to peroxisomes in mammalian cells, plants, insects and yeasts (Gould *et al.*, 1990a). PTS1 variants have been shown to function in several different organisms (Aitchison *et al.*, 1991; Keller *et al.*, 1991; Subramani, 1993). However, several peroxisomal proteins lack this signal sequence. One of these is the long-chain 3-oxoacyl-CoA thiolase which catalyses the last step of the peroxisomal fatty acid β -oxidation cycle. So far the corresponding gene has been cloned in man (Bout *et al.*, 1988), rat (Hijikata *et al.*, 1990), cucumber (Preisig-Müller and Kindl, 1993), *Saccharomyces cerevisiae* (Erdmann, 1989; Igual *et al.*, 1991) and *Yarrowia lipolytica* (Berninger *et al.*, 1993).

For the two peroxisomal thiolases from rat (A and B) it was demonstrated that they are synthesized as larger precursors with amino-terminal prepieces which are cleaved upon translocation of the proteins into peroxisomes (Hijikata *et al.*, 1987; Bodnar and Rachubinski, 1990). Moreover, it was shown that the targeting signal of rat thiolase B resides within its amino-terminal prepiece. In fact, the first 11 amino acids of rat thiolase B are sufficient to target an otherwise cytosolic passenger protein to peroxisomes (Osumi *et al.*, 1991; Swinkels *et al.*, 1991). Similarly, the amino-terminal 16 amino acids of *S.cerevisiae* thiolase are necessary and sufficient for the peroxisomal targeting of the enzyme (Erdmann, 1994; Glover *et al.*, 1994). This second peroxisomal targeting signal was named PTS2. Several other peroxisomal matrix proteins contain sequences similar to the PTS2 identified in thiolases of rat and yeast, including human and plant thiolases (Bout *et al.*, 1988; Preisig-Müller and Kindl, 1993) as well as glyoxysomal malate dehydrogenase of watermelon (Gietl, 1990), amine oxidase of *Hansenula polymorpha* (Bruinenberg *et al.*, 1989) and aldolase of *Trypanosoma brucei* (Blattner *et al.*, 1992). For two of these proteins, malate dehydrogenase (Gietl *et al.*, 1994) and aldolase (Blattner *et al.*, 1992), it could be shown that these sequences indeed serve as PTSs. The accumulated data suggest that there are at least two different signal sequences, PTS1 and PTS2, involved in the targeting of proteins to peroxisomes.

To understand the mechanisms underlying such sorting processes, it is critical to identify the cellular machinery functioning to recognize and import proteins. We (Erdmann *et al.*, 1989; Erdmann and Kunau, 1992; Höhfeld *et al.*, 1992) and other laboratories (van der Leij *et al.*, 1992; Elgersma *et al.*, 1993; Zhang *et al.*, 1993) have shown that it is possible to apply genetics to the biogenesis of peroxisomes in the yeast *S.cerevisiae*. This approach has led to the identification of at least 15 complementation groups comprising mutants impaired in either peroxisome biogenesis or proliferation. Recently, mutations affecting peroxisomal biogenesis have also been isolated from other yeast species (reviewed in Lazarow, 1993).

We have analysed our *pas* mutants for import defects and present genetic evidence for the existence of two import pathways for peroxisomal matrix proteins. Mutant *pas7-1* exhibits a selective import defect for thiolase while SKL-containing proteins are properly imported. Furthermore, we report the molecular cloning of *PAS7*, an initial characterization of its gene product, Pas7p, and propose that it mediates the binding of thiolase to peroxisomes.

Results

Isolation and characterization of the *pas7-1* mutant

We have previously described the isolation of peroxisomal mutants of *S.cerevisiae* characterized by their inability to grow on oleic acid [oleic acid non-utilizing (*onu*) phenotype; Erdmann *et al.*, 1989]. Among these strains two principally different classes of peroxisomal mutants could be distinguished: *fox* and *pas* mutants. The *fox* mutants (fatty acid oxidation) are characterized by a deficiency of one of the individual β -oxidation proteins, whereas the *pas* mutants are affected in peroxisome assembly.

To obtain additional *pas* mutants we have selected *onu* strains as described previously (Erdmann *et al.*, 1989). Among them were mutants *pas7-1* (Figure 1) and *pas7-2*. Genetic analysis demonstrated that these mutants define a new complementation group. The meiotic segregation behaviour revealed that a single mutant gene was responsible for preventing growth of *pas7-1* and *pas7-2* on oleic acid. Backcrosses of the mutant strains to wild-type cells yielded diploids that were able to utilize oleic acid; therefore, the mutations of *pas7-1* and *pas7-2* are recessive. Further work was focused on the *pas7-1* mutant.

Determination of peroxisomal enzyme activities in whole cell lysates of *pas7-1* excluded a deficiency of a β -oxidation enzyme activity and hence a *fox* mutation because peroxisomal matrix enzymes could be induced almost to wild-type levels (data not shown). These results suggested that the *onu* phenotype of *pas7-1* is due to a defect in peroxisome assembly. We therefore expected to obtain a mislocalization of the peroxisomal enzymes during sedimentation analysis as it had been previously observed for the *pas* mutants *pas1-pas3*. Of all tested peroxisomal enzymes of *pas7-1*, only thiolase was predominantly detectable in the soluble fraction suggesting that this enzyme is cytosolic rather than particulate (Table I). This conclusion was confirmed by more detailed fractionation studies using sucrose density gradient centrifugation. The sedimentation pattern of the marker enzymes catalase and cytochrome *c* oxidase indicated well-separated peroxisomes (1.22 g/cm³) and mitochondria (1.18 g/cm³) in control wild-type (Figure 2A) as well as *pas7-1* cells (Figure 2B). However, in contrast to the wild-type situation, peroxisomes of *pas7-1* cells did not contain 3-oxoacyl-CoA thiolase. This enzyme migrated at a density of 1.11 g/cm³ indicating its cytosolic nature (Figure 2B).

The gradient fractions were further analysed by Western blot analysis using polyclonal antibodies against 3-oxoacyl-CoA thiolase. As shown in Figure 2B, thiolase could only be detected in low density fractions of mutant

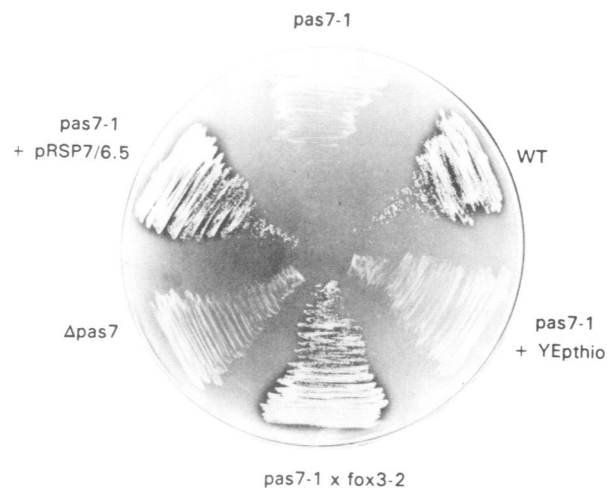


Fig. 1. Growth behaviour of different strains on oleic acid medium (YNO agar). In contrast to the wild-type strain, the *pas7-1* mutant and the *pas7* null mutant are not able to grow on a YNO agar plate. Transformation of *pas7-1* with plasmid pRSP7/6.5 results in a functional complementation of the oleic acid non-utilizing phenotype, whereas expression of thiolase from a multicopy plasmid (YEpthio) cannot rescue the mutant. A diploid strain obtained after crossing of *pas7-1* and *fox3-2* mutant cells is able to utilize oleic acid, indicating that both mutants belong to different complementation groups. Growth on YNO agar is indicated by the appearance of single cell colonies and a typical 'halo'. The plate shown was incubated at 30°C for 7 days.

Table I. Distribution pattern of peroxisomal and mitochondrial marker enzymes in the 25 000 g supernatant and pellet fractions of cell lysates from wild-type, *pas7* mutants and *pas7* transformants grown for 18 h on YNO medium

Strain	Enzyme	Activity (nkat) in 25 000 g		
		Supernatant fraction (A1)	Pellet fraction (A2)	A1/A2
Wild-type	Thiolase	36.7	95.0	0.4
	Catalase	27.5×10 ³	150.0×10 ³	0.2
	Epimerase	23.3	115.0	0.2
	Fumarase	43.3	108.4	0.4
<i>pas7-1</i>	Thiolase	153.4	10.0	15.3
	Catalase	10.7×10 ³	69.5×10 ³	0.2
	Epimerase	20.0	66.7	0.3
	Fumarase	30.0	108.4	0.3
<i>pas7-1</i> + pRSP7/1.2	Thiolase	7.2	10.0	0.7
	Catalase	10.8×10 ³	47.9×10 ³	0.2
	Epimerase	13.3	27.5	0.5
	Fumarase	35.0	73.3	0.5
<i>pas7-1</i> + YEpthio	Thiolase	3.3×10 ³	350.1	9.5
	Catalase	10.8×10 ³	23.6×10 ³	0.5
	Epimerase	8.3	13.3	0.6
	Fumarase	11.7	25.0	0.5
Δ <i>pas7</i>	Thiolase	111.7	13.3	8.4
	Catalase	21.3×10 ³	52.8×10 ³	0.4
	Epimerase	8.3	31.7	0.3
	Fumarase	20.0	36.7	0.5

cells whereas in wild-type cells this protein was primarily found in the peroxisomal fractions (Figure 2A).

Morphological characterization of oleic acid induced *pas7-1* cells by electron microscopy and immunocytochemistry confirmed the biochemical results. These studies demonstrated that the mutant contained normal-looking

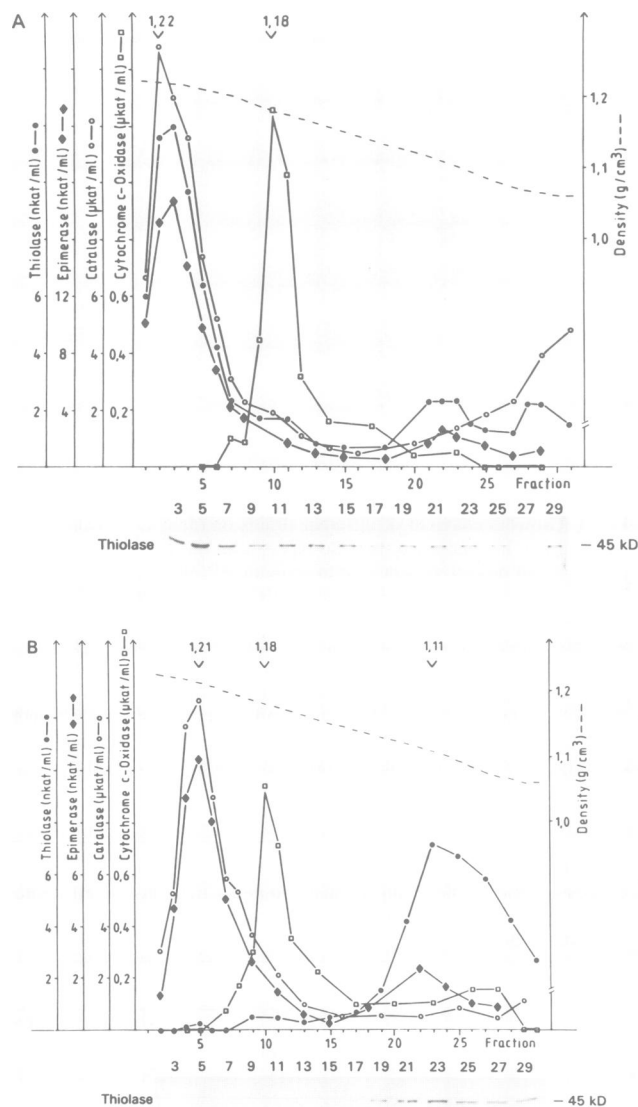


Fig. 2. Activities of peroxisomal and mitochondrial marker enzymes in fractions of a continuous 20–53% sucrose density gradient of cell lysates of wild-type (A) and *pas7-1* (B). Densities at activity peaks are indicated. The fractions were also checked immunologically for the presence of thiolase. 0.1% of the volume of each fraction was loaded per lane.

peroxisomes lacking immunocytochemically detectable thiolase, which was exclusively found in the cytoplasm (Figure 3A). Hence, biochemical and morphological data clearly show a specific mislocalization of thiolase to the cytoplasm of *pas7-1* cells.

Mislocalization of thiolase is not due to a mutation of its structural gene

The biochemical and morphological data were in accordance with the possibility that *pas7-1* contains a mutation of the thiolase gene (*FOX3* gene of *S.cerevisiae*) which does not affect the activity of the enzyme but the import of thiolase into peroxisomes. To investigate this question, *pas7-1* cells were crossed with mutants of the *fox3* complementation group (defect in the structural gene of 3-oxoacyl CoA thiolase). The crosses exclusively yielded

diploids that regained the ability to utilize oleic acid as a carbon source (Figure 1) clearly indicating that a protein other than thiolase is affected in *pas7-1*.

Further evidence was provided when the wild-type copy of *FOX3* was expressed from a multicopy vector in *pas7-1* cells. The transformation neither led to functional complementation of the mutant strain (Figure 1) nor to import of thiolase into peroxisomes. After differential centrifugation of lysates of the transformant *pas7-1*+YEthio thiolase was still predominantly detected in the 25 000 g supernatant (Table I). Sucrose gradient fractionation further stressed the exclusive presence of thiolase in the cytosol (data not shown). These observations were confirmed by immunocytochemistry: the over-expressed thiolase had accumulated in the cytosol and the nucleus, but was absent from peroxisomes (Figure 3D). These findings demonstrate that the *pas7-1* mutation does not affect 3-oxoacyl-CoA thiolase itself but a protein essential for import of thiolase into peroxisomes.

Cloning of the PAS7 gene by functional complementation

The recessive and monogenic nature of the *pas7-1* mutation opened the way for cloning *PAS7* by functional complementation. *pas7-1* cells were transformed with a genomic library of *S.cerevisiae* maintained in the vector YEp13 (Broach *et al.*, 1979). Transformed cells were selected for leucine prototrophy and subsequently screened for growth on oleic acid medium. Among 20 000 transformants two clones were recovered that had regained the ability to utilize oleic acid as carbon source. Restriction analysis of the isolated plasmids from these two transformants revealed that they contained identical inserts of 10 kb in size. In order to localize *PAS7*, subclones were constructed in pRS316 (Sikorski and Hieter, 1989) by using a 6.5 kb *Sall*–*XbaI* fragment (pRSP7/6.5; Figure 4) of the original plasmid and fragments obtained through partial digestion with *Sau3AI*. These subclones were tested for their ability to complement mutant strain *pas7-1*. The smallest genomic *Sau3AI* fragment able to complement comprised a region of 1.26 kb (pRSP7/1.2; Figure 4). *pas7-1* cells transformed with this fragment had lost their *pas* phenotype and regained the ability to grow on oleic acid agar plates. Thiolase activity of the transformants was predominantly found in the particulate fraction (Table I) and the protein was located in peroxisomes by means of immunocytochemistry (data not shown).

Sequence analysis of PAS7

The nucleotide sequence of *PAS7* was determined by sequencing a series of nested *Bal31*-generated deletion templates and defined restriction fragments using the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977). The sequenced stretch of 1478 bp contains an open reading frame of 1125 bp (Figure 5). A 1.6 kb transcript was detected by Northern blot analysis in both glucose-repressed and oleate-induced cells (data not shown). The open reading frame of *PAS7* encodes a potential polypeptide of 375 amino acids with a predicted molecular mass of 42.3 kDa.

Surprisingly, the complementing fragment pRSP7/1.2 contains only 53 bp 5' of the ORF. A search for sequence

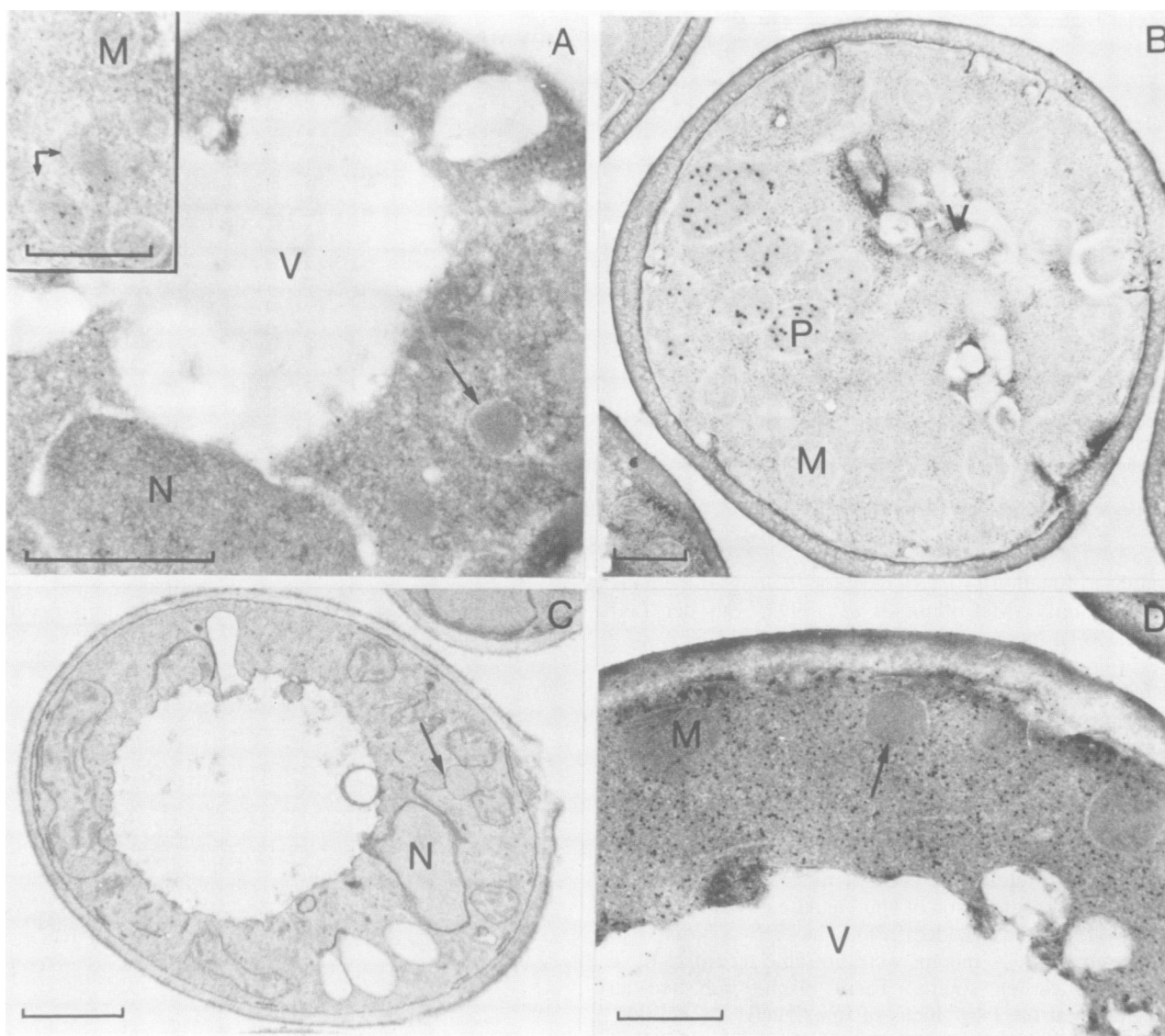


Fig. 3. (A) Immunocytochemical detection of thiolase and SKL-containing proteins in oleate-induced cells of mutant *pas7-1*. In these cells thiolase is located in the cytosol, whereas SKL proteins can be detected within peroxisomes (inset A). (B) Detection of thiolase in cells of the *pas7* null mutant transformed with a single copy plasmid (pRSmycP7) expressing Myc-tagged Pas7 protein under the control of the *CUP1* promoter. Thiolase labelling is found almost exclusively in peroxisomes. (C) After induction on oleic acid medium $\Delta pas7$ contains peroxisomes of normal morphology. (D) Immunogold labelling for thiolase in *pas7-1* cells overexpressing thiolase is found all over the cytosol, but not in peroxisomes. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bars represent 0.5 μm . Peroxisomes are indicated by arrows.

homology using the EMBL Nucleotide Sequence Database (release 38) revealed no significant sequence similarity to any other known protein. A hydropathy analysis showed that the Pas7 protein is largely hydrophilic with no apparent hydrophobic domains with the potential to span a membrane (data not shown). A more detailed analysis of the Pas7p primary sequence showed that it contains a repeated motif, referred to as the WD-40 repeat (van der Voorn and Ploegh, 1992). The WD-40 motif consists of ~40 amino acid residues with a characteristic tryptophan–aspartic acid pair initially identified within the β -subunit of the heterotrimeric G protein transducin (Fong *et al.*, 1986). Such a repetitive domain has been shown to exist in a number of other proteins involved in diverse cellular processes including mitosis (Cdc4p and Cdc20p), signal transduction (Ste4p), mRNA splicing (Prp4p and Prp17p) and transcription regulation (Tup1p) (for a review see van

der Voorn and Ploegh, 1992). The WD-40 repeat can be divided into two relatively conserved elements (A and B) which are spaced by regions variable in both length and sequence. Based on the consensus amino acid sequence defined by van der Voorn and Ploegh (1992), the Pas7 protein contains five repeats of element B of the WD-40 motif (Figure 6), whereas part A could not be identified. This is also the case for some other members (e.g. Cdc20p and Pwp1p) and led to the suggestion that part A may be dispensable (van der Voorn and Ploegh, 1992). The function of the motif is not known for any of the WD-40 proteins but some of them have been found to be functionally related to members of another family of repeat-containing proteins: the tetratricopeptide (TPR) gene family (for a review see Goebel and Yanagida, 1991). Interestingly, the Pas10 protein of *S.cerevisiae* and its homologue from *Pichia pastoris*, Pas8p, which are

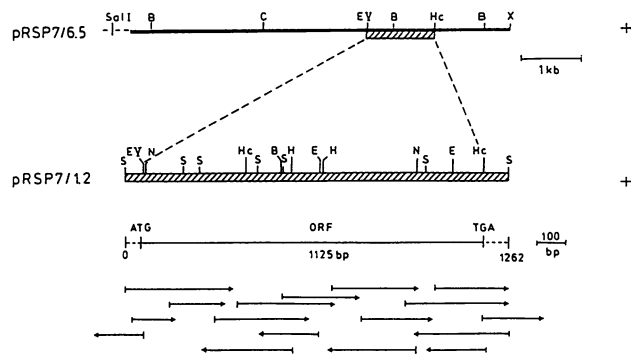


Fig. 4. Localization and sequencing strategy of the *pas7* complementing region. The solid black line indicates the 6.5 kb genomic fragment of plasmid pRSP7/6.5. A detailed restriction endonuclease map of the smallest complementing *Sau3A*I fragment (contained in plasmid pRSP7/1.2) and the identified open reading frame is given in the lower panel. Arrows indicate the direction and extent of sequence determinations. The complementing ability of the plasmids is denoted by a plus sign. B, *Bam*HI; C, *Clal*; E, *Eco*RI; EY, *Eco*RV; H, *Hind*III; Hc, *Hinc*II; N, *Nde*I; S, *Sau*3A1; X, *Xba*I.

thought to function as PTS1 receptors belong to the TPR protein family (McCollum *et al.*, 1993; van der Leij *et al.*, 1993).

Deletion of the *PAS7* locus

To confirm the authenticity of *PAS7* and to exclude the possibility that a suppressor locus was cloned, we created a null allele by deleting the chromosomal copy of a wild-type strain. Cells of the resultant *pas7* null mutant ($\Delta pas7$) acquired the *onu* phenotype (Figure 1). $\Delta pas7$ was mated to a wild-type strain, diploids were sporulated and asci dissected into tetrads. In each of the 22 asci examined, an absolute cosegregation of *onu*⁻ and *Leu*⁺ was observed. Moreover, diploids yielded from a backcross of $\Delta pas7$ to the original *pas7-1* mutant were not able to utilize oleic acid as a carbon source. These results indicated an integration/deletion event was confirmed by Southern blot analysis (data not shown). Taken together these data demonstrate that the authentic *PAS7* gene has been cloned.

$\Delta pas7$ cells exhibited the same biochemical and morphological phenotype as the original *pas7-1* mutant. As expected, $\Delta pas7$ cells mislocalize thiolase to the cytosol (Table I). This result was also confirmed by immunogold labelling and immunofluorescence microscopy (data not shown). Introduction of the wild-type copy of *PAS7* completely corrected the thiolase import defect (data not shown). $\Delta pas7$ cells also possess morphologically normal peroxisomes (Figure 3C).

Import of PTS1-containing proteins is not affected in *pas7* cells

To establish whether *Pas7p* is required for import of PTS1-containing proteins, we investigated the intracellular localization of proteins possessing an SKL tripeptide at their extreme C-terminus in *pas7* cells of the original and null mutant. The SKL motif has been identified as the minimal targeting signal of a number of peroxisomal proteins from various organisms (Gould *et al.*, 1990a). In *S.cerevisiae* five known peroxisomal proteins possess an SKL at their carboxy-termini (Kunau and Hartig, 1992). One of those is the multifunctional β -oxidation protein

-149	AACACAAACTGTGTTTTCTTACCATAAAATC
-118	TTTTACCGGAGGGTCGAAATCGTTTTCTTTCTGATTGGAATCGAACGCTCGGAAAAG
-59	TGCAGAGATCTCATATTGTTAACGGACTATCATCTAACTTTTGCATAATTTATACAAC
+1	ATG CTC AGA TAT CAT ATG CAA GGT TTT AGT GGG TAC GGT GTC CAG Met Leu Arg Tyr His Met Gln Gly Phe Ser Gly Tyr Gly Val Gln
+46	TAT TCT CCC TTT TTC GAT AAC AGG CTC GCG GTA GCT GGT GGT TCA Tyr Ser Pro Phe Phe Asp Asn Arg Leu Ala Val Ala Ala Gly Ser
+91	AAC TTT GGC CTG GTT GGG AAT GGA AAA TTG TTC ATC CTT GAG ATT Asn Phe Gly Leu Val Gly Asn Gly Lys Leu Phe Ile Leu Glu Ile
+136	GAT CGT TCA GGT AGA ATA GTG GAA GTT AAT TCC TTT TTA ACA CAG Asp Arg Ser Gly Arg Ile Val Glu Val Asn Ser Phe Leu Thr Gln
+181	GAT TGT TTA TTT GAT CTT GCA TGG AAC GAA AGT CAT GAA AAC CAA Asp Cys Leu Phe Asp Leu Ala Trp Asn Glu Ser His Glu Asn Gln
+226	GTG TTG GTT GCA CAG GGC GAT GGT ACA TTA CGC TTG TTT GAT ACA Leu Val Ala Ala Gln Gly Asp Gly Thr Leu Leu Phe Asp Thr
+271	ACC TTT AAA GAG TTT CCT ATT GCT ATA TTT AAA GAG CAT GAA CGA Thr Phe Lys Glu Phe Pro Ile Ala Ile Phe Lys Glu His Glu Arg
+316	GAA GTA TTC AGT TGT AAT TGG AAC TTA GTC AAC AGG CAG AAT TTC Glu Val Phe Ser Cys Asn Trp Asn Leu Val Asn Arg Leu Thr Gln
+361	TTA AGT AGT TCA TGG GAT GGA TCT ATA AAA ATA TGG TCC CCT CTA Leu Ser Ser Ser Trp Asp Gly Ser Ile Lys Ile Trp Ser Pro Leu
+406	AGA AAG CAA AGT TTA ATG ACC CTT ACT CCA CGA CCT TTA GAG ATT Arg Lys Gln Ser Leu Met Thr Leu Thr Pro Arg Pro Leu Glu Ile
+451	ACC AAA ATG GTG GAT CCA TTA AAC GCC ATT ATA TTG AAA AAG AAA Thr Lys Met Val Asp Pro Leu Asn Ala Ile Ile Leu Lys Lys Lys
+496	AGC TTT ACA GGT ATT TCA AAA AAC AGG AAC TGT GTA TAC CAA GCA Ser Phe Thr Gly Ile Ser Lys Asn Arg Asn Cys Val Tyr Gln Ala
+541	CAG TTC TCG CCC CAC GAC CAA AAT CTC GTA TTA TCC TGT TCA GGG Gln Phe Ser Pro His Asp Gln Asn Leu Val Leu Ser Cys Ser Gly
+586	AAT TCT TAT GCA AGC TTA TTT GAC ATT AGA CTA CCT TCC GGC AAA Asn Ser Tyr Ala Ser Leu Phe Asp Ile Arg Leu Pro Ser Gly Lys
+631	AAT CAG AAT AAT TTT TTA GTG CAT TCA GGA CTA GAA GCA TTG ACT Asn Gln Asn Asn Phe Leu Val His Ser Gly Leu Glu Ala Leu Thr
+676	TGC GAT TTC AAC AAA TAC AGA CCT TAT GTA GTT GCC ACA GGA GTT Cys Asp Phe Asn Lys Tyr Arg Pro Tyr Val Val Ala Thr Gly Gly
+721	GTA GAT AAT GCC ATT AGA ATC TGG GAC ATT AGG ATG CTA AAT AAA Ile Asp Asn Ala Ile Arg Ile Trp Asp Ile Arg Met Leu Asn Lys
+766	AAT GAA TCA GCG ACT ATC AAG AGG ACT GTG CCC GGC CAA CTT CAC Asn Glu Ser Ala Thr Ile Lys Arg Thr Val Pro Gly Gln Leu His
+811	AAT TCA TCA TGC ATC AAT GAA ATC CCT AAT GCG CAC GGG CTG GCA Asn Ser Ser Cys Ile Asn Glu Ile Pro Asn Ala His Gly Leu Ala
+856	ATC AGA AAA GTT ACC TGG TCC CCT CAT CAT TCC AAT ATT TTA ATG Ile Arg Lys Val Thr Trp Ser Pro His Ser Asn Ile Leu Met
+901	TCA GCT TCA TAT GAT ATG ACC TGT CGA ATA TGG AGA GAT CTC AGC Ser Ala Ser Tyr Asp Met Thr Cys Arg Ile Trp Arg Asp Leu Ser
+946	AAC GAT GGT GCA AAA GAA ACA TAT AAA ACT ACG TCT ACG GAT GCT Asn Asp Gly Ala Lys Glu Thr Tyr Lys Thr Asn Ser Thr Asp Ala
+991	ACT AAA GGT TCC ATT TTC AAT TTC ACA CAG CAT TCA GAA TTC GTA Thr Lys Gly Ser Ile Phe Asn Phe Thr Asn Phe Thr Ser Glu Phe Val
+1036	TTT GGA GCT GAC TGG AGC TTG TGG GGA AAG CCA GGA TAT GTG GCT Phe Gly Ala Asp Trp Ser Leu Trp Gly Lys Pro Gly Tyr Val Ala
+1081	TCA ACT GCA TGG GAT GGA AAT TTA TTT GTA TGG AAC GGC TTA GGT Ser Thr Ala Trp Asp Gly Asn Leu Phe Val Trp Asn Gly Leu Gly
+1126	TGA CACATATTCCTTTTACACAAATTTTTGCGATTATTTAAACTTTAAATATTTTT ***
+1184	CAATCATTGTCAGCAATTGGCAGATCATTTACGTAATTATTTAATTTCTCAACACGAA
+1243	ATTATGGGTAACAACGCTTCTGACTGTGCAAAAAGAACATTTCTAAAAACATACCTACGG
+1302	CAAAAAGTCTAATTACTCAAATTAATC

Fig. 5. Nucleotide sequence of *PAS7* and deduced primary sequence of the gene product. The smallest complementing *Sau3A* subclone starts at position -53 of the upstream region. These sequence data are available from the EMBL database under accession number X81424.

Fox2p (Hiltunen *et al.*, 1992). Subcellular fractionation studies showed that one of the enzymatic activities of Fox2p [3-hydroxyacyl-CoA epimerase, the result of two different enoyl-CoA hydratases (Hiltunen *et al.*, 1992)] was particulate in the *pas7-1* mutant (Table I) and showed a parallel distribution to catalase activity in sucrose density gradients (Figure 2B), indicating that the protein was correctly imported into peroxisomes.

This conclusion was supported and extended to other SKL-containing proteins by results of immunological analysis using antibodies raised against a synthetic peptide containing one version of the minimal peroxisomal targeting signal (serine-lysine-leucine-COOH; Gould *et al.*, 1990b).

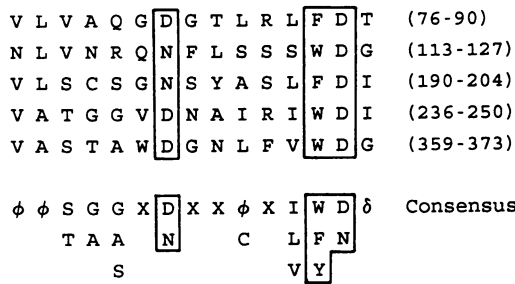


Fig. 6. Pas7p is a member of the β -transducin-related protein family. Alignment of the five repeats of the second half (segment B) of the WD-40 motif present in the Pas7p sequence (the respective regions of the primary sequence are given in brackets). Those amino acids which are highly conserved among all proteins of the WD-40 family (van der Voorn and Ploegh, 1992) are boxed. x, any amino acid; ϕ , hydrophobic residues preferred; δ , non-charged amino acid.

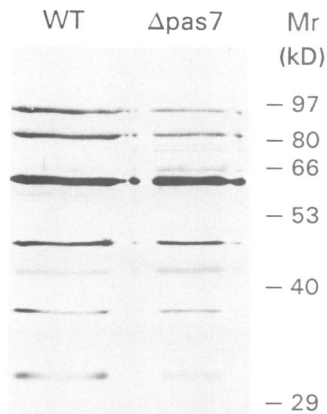


Fig. 7. Immunological detection of SKL-containing polypeptides in peroxisomes from oleate-induced wild-type and *pas7* null mutant cells using an anti-SKL antiserum. The same set of seven major immunoreactive bands was recognized in both wild-type and $\Delta pas7$ peroxisomes. Equal amounts of protein (50 μ g) were loaded in each lane.

Immunogold labelling with these antibodies revealed that SKL proteins are located in peroxisomes of *pas7-1* cells (see inset in Figure 3A). In addition, peroxisomal peak fractions of sucrose density gradients from wild-type and $\Delta pas7$ cells were subjected to SDS-PAGE and Western blot analysis with the anti-SKL antibody (Figure 7). Since the identical pattern of seven immunoreactive polypeptides was observed in the peroxisomes of both strains, we conclude that the SKL-dependent import pathway is not impaired in $\Delta pas7$.

Peroxisomes from $\Delta pas7$ cells seem to lack only thiolase

To investigate if oleate-induced cells of $\Delta pas7$ fail to import other proteins besides thiolase, we purified peroxisomes from both $\Delta pas7$ and wild-type cells using Nycodenz density gradients and analysed them by SDS-PAGE. The advantages of using Nycodenz rather than sucrose density gradients for isolation of highly purified peroxisomes has been established previously (Kunau *et al.*, 1993). Comparison of the resulting poly-

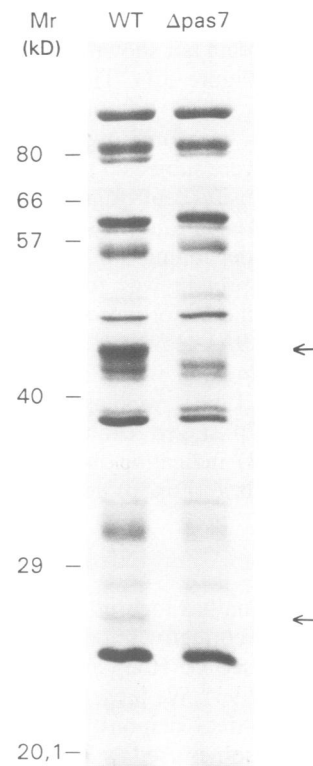


Fig. 8. SDS-PAGE of peroxisomes from oleate-induced wild-type and *pas7* null mutant cells. Organelles were purified by Nycodenz gradient centrifugation. Equal amounts of protein (5 μ g) were loaded per lane and analysed by silver staining.

peptide pattern indicates that peroxisomes of both type of cells have almost identical protein compositions (Figure 8). Notable exceptions were the thiolase band of M_r 45 kDa (Erdmann and Kunau, 1994) and an unknown polypeptide of M_r 26 kDa. Western blot analysis revealed that the 26 kDa band is not a degradation product of thiolase (data not shown). Both polypeptides were missing in peroxisomes of cells deficient in Pas7p; however, while the thiolase subunit was consistently absent in all repetitions of this experiment the distribution of the 26 kDa band was less uniform. The reasons for this observation are presently unknown.

The results presented are consistent with the fact that up to now thiolase is the only peroxisomal protein of *S.cerevisiae* known to possess a PTS2. However, the results do not exclude the possibility that import of other proteins not detected or resolved by SDS-PAGE analysis may be Pas7p-dependent.

Myc-tagged Pas7p partially localizes to peroxisomes

To determine the location of Pas7p in the yeast cell, a fusion protein was constructed in which the amino-terminus of Pas7p was tagged with a human c-Myc epitope (Evan *et al.*, 1985). In addition, the chimeric protein was placed under the control of the strong, inducible *CUP1* promoter (Butt *et al.*, 1984; Karin *et al.*, 1984) in either the *CEN* vector pRS316 (pRSmycP7) or the 2 μ vector YE352 (YEpmycP7). Both plasmids encoded a fusion protein of M_r 44.5 kDa which could be detected in $\Delta pas7$ cells with a monoclonal antibody against the Myc epitope and which complemented the thiolase import deficiency

of these cells. The transformants grew on oleate as the sole carbon source (data not shown) and imported thiolase into peroxisomes (Figure 3B). Thus, the epitope-tagged Pas7p is functional.

When $\Delta pas7$ cells transformed with either the single or multicopy plasmid were subjected to subcellular fractionation by sucrose density centrifugation Pas7p showed a dual distribution. Using anti-Myc antibodies a minor portion (~5–15%, depending on the plasmid used) of the epitope-tagged protein was found comigrating with peroxisomes while the major part was detected on top of the gradient (Figure 9A). However, the result was different when the *fox3-2A* mutant, which is deficient in thiolase protein (Erdmann, 1994), was used instead of $\Delta pas7$. In these cells Pas7p stayed exclusively on top of the gradient (Figure 9B) indicating that its association with peroxisomes is thiolase (Fox3p)-dependent.

Discussion

Considerable attention has focused on the mechanisms by which proteins are correctly imported into peroxisomes (for recent reviews see De Hoop and Ab, 1992; Lazarow, 1993; Subramani, 1993). We present genetic, biochemical and morphological data demonstrating that the import pathway into peroxisomes used by 3-oxoacyl-CoA thiolase in *S.cerevisiae* is different from that of SKL-containing proteins. *pas7* mutants contain recessive mutations that lead to the cytosolic accumulation of 3-oxoacyl-CoA thiolase (Table I, Figures 2B and 3A). This study shows that these mutations affect a novel gene, *PAS7*, which is essential for import of thiolase.

Which proteins are dependent on Pas7p for their import into peroxisomes?

Peroxisomes of both $\Delta pas7$ and wild-type cells exhibit the same morphology, migrate to the same density in sucrose gradients and show almost the same polypeptide pattern when analysed by SDS-PAGE. However, two polypeptides were missing in silver-stained peroxisomal fractions of $\Delta pas7$ cells (Figure 8): thiolase and a 26 kDa polypeptide. Since the latter polypeptide is not consistently absent and is as yet entirely unknown, further studies will have to substantiate the possibility that this is a second candidate for a Pas7p-dependent import. Since thiolase is the only protein known in *S.cerevisiae* to contain PTS2, we cannot determine whether Pas7p is specific for thiolase or whether it recognizes PTS2. Expression of a heterologous PTS2-containing protein will be used to address this question.

Our results clearly show that all SKL-containing proteins detectable with antibodies directed against the SKL signal do not depend on Pas7p to reach the peroxisomal matrix (Figures 3A and 7). This indicates that the PTS1 import pathway in both cell types is intact.

Other genetic evidence also points to the existence of at least two distinct signal-dependent pathways for import of matrix proteins into peroxisomes of yeasts as well as of higher eukaryotes. It has been reported that PTS1-containing catalase, luciferase and Fox2p did not enter the reticulum-like peroxisomal membrane structure of the *pas10* mutant of *S.cerevisiae*, whereas thiolase was found to be peroxisomal (van der Leij *et al.*, 1992; T.Franken

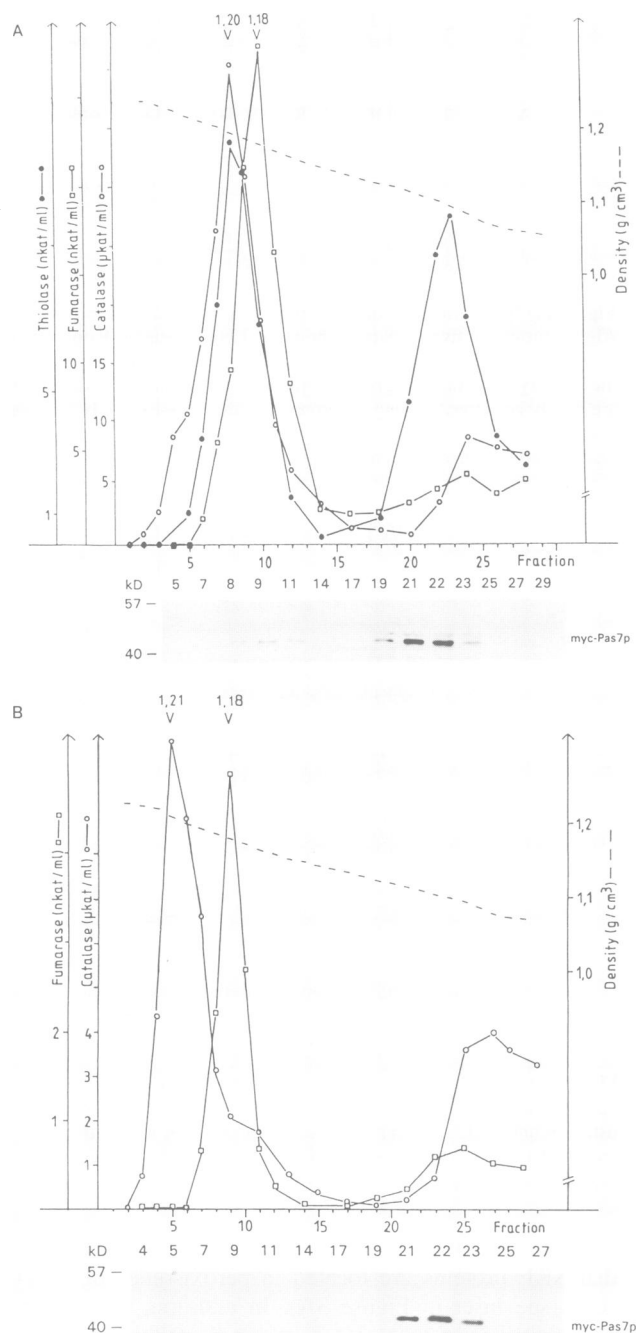


Fig. 9. Immunological detection of Myc-Pas7p in fractions of a continuous 20–53% sucrose density gradient of cell lysates of the *pas7* null mutant (A) and the *fox3-2A* mutant (B) transformed with a single copy plasmid expressing Myc-Pas7p under control of the *CUP1* promoter. 2% of the volume of each fraction was loaded per lane. Immunoreactive proteins were visualized by the ECL system.

and W.-H.Kunau, unpublished results). Furthermore, Zhang *et al.* (1993) recently described another peroxisomal mutant of *S.cerevisiae*, *peb5*, which is defective in catalase import but possesses peroxisomes containing thiolase. Moreover, certain complementation groups of Zellweger syndrome fibroblasts are impaired in the transport of proteins containing PTS1, but not PTS2, into peroxisomes (Balfe *et al.*, 1990; Walton *et al.*, 1992). Conversely, RCDP fibroblasts are reported to contain cytosolic thiolase while other peroxisomal matrix proteins are particulate

(Balfe *et al.*, 1990). This is consistent with the recent finding (Motley *et al.*, 1994) that two out of five complementation groups (Shimozawa *et al.*, 1993) of fibroblasts from patients suffering from peroxisomal assembly disorders show differential import deficiencies. These examples underline the notion that the same basic principles seem to underlie peroxisomal protein import in lower and higher eukaryotes.

Another intriguing problem related to import of peroxisomal matrix proteins is the question as to the existence of a third pathway. Acyl-CoA oxidase of *S.cerevisiae* (Dmochowska *et al.*, 1988), the first and rate-limiting enzyme of the β -oxidation cycle, seems to have neither a PTS1 nor a PTS2. Moreover, the same enzyme of another yeast, *Candida tropicalis*, was reported to contain two internal import signals (Small *et al.*, 1988). However, in *pas7* mutants acyl-CoA oxidase behaves like the PTS1 proteins (data not shown). Recently, catalase was reported to possess a SKL-type as well as an additional internal targeting signal (Kragler *et al.*, 1993), but again also catalase behaves like PTS1 proteins in *pas10* mutant cells. There are several possibilities to account for these observations: Pas10p does not specifically recognize PTS1, the redundant targeting information is not functionally active in the context of the complete catalase sequence or an SKL-containing protein is essential for the recognition of the internal signals.

What is the function of Pas7p?

The most simple explanation for the data described would be that Pas7p is a receptor which specifically binds the PTS2 of thiolase and directs it to the translocation machinery. A similar role has been postulated for Pas10p of *S.cerevisiae* (Van der Leij *et al.*, 1993) and Pas8p of *P.pastoris* (McCollum *et al.*, 1993) in the SKL (PTS1) import pathway. The existence of two distinct types of targeting signals and receptors in one organelle has a parallel in the import of proteins into mitochondria (Pfanner *et al.*, 1991). However, Myc-tagged Pas7p, unlike MOM19 and MOM72, is a hydrophilic protein which is predominantly found in the cytosol and only a minor portion of it comigrates with peroxisomes in sucrose gradients (Figure 9A). This seems to argue against the notion that Pas7p functions as a receptor for thiolase. However, it should be noted that *PAS7* has been expressed from the *CUP1* promoter. To the best of our current knowledge the *CUP1* promoter is stronger than the *PAS7* one. Therefore it is difficult to estimate how much Pas7p would be found soluble under physiological conditions. Moreover, the most interesting aspect of our results is the unexpected observation that the association of this minor part of Pas7p with peroxisomes is thiolase-dependent. In *fox3-2* cells expressing no thiolase, Pas7p is exclusively found on top of the gradient (Figure 9B).

On the basis of the presented results, we speculate that cytosolic Pas7p might direct thiolase to peroxisomes by shuttling between cytosol and peroxisomal membranes (Figure 10). Cytosolic Pas7p could recognize thiolase and only the complex of these two proteins would bind to the peroxisomal membrane. Subsequently, thiolase might be directed to the translocation machinery and Pas7p would be released to the cytosol. This hypothetical model contains two genetically and biochemically testable predictions.

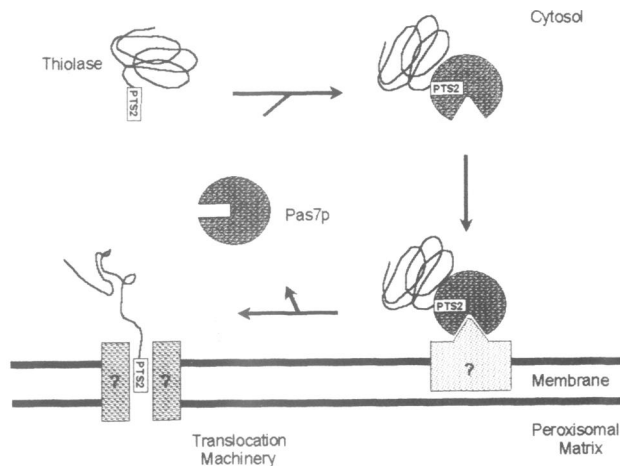


Fig. 10. Hypothetical model of Pas7p function. See text for details.

First, thiolase and Pas7p interact physically. Secondly, there is a component of the peroxisomal membrane which binds the thiolase–Pas7p complex. Pas7p contains the WD-40 motif which has been reported to interact with proteins containing a TPR motif (Goebel and Yanagida, 1991; van der Voorn and Ploegh, 1992). A TPR-containing Pas protein, Pas10p, has been identified. However, genetic evidence seems to exclude (see above) the interaction of Pas7p and Pas10p, the two proteins functionally critical for the PTS1 and PTS2 pathway, respectively.

The proposed model is not without precedents: for example, SecB (Wickner *et al.*, 1991) and SRPs (Luirink and Dobberstein, 1994) fulfil similar roles as discussed here for Pas7p. Recently, it has been predicted that such cytosolic factors should exist for all organelles to promote the specificity of protein targeting by preventing the interaction of preproteins with improper membrane surfaces (Lithgow *et al.*, 1993).

For such a role Pas7p must recognize the targeting sequence of thiolase. If this is so, it seems reasonable to assume that Pas7p should also bind other PTS2-containing proteins and thus might represent a PTS2 receptor.

An alternative possibility would be that Pas7p does not act as a receptor but rather as a thiolase-specific chaperone. Pas7p could maintain thiolase in or convert it into an import-competent form. Currently we are extending our analysis of Pas7p function to distinguish between these different possibilities.

Materials and methods

Strains and media

Yeast and bacterial strains used in this study are listed in Table II. Yeast complete and minimal media have been described earlier (Erdmann *et al.*, 1989). YNO medium contained 0.1% oleic acid, 0.05% Tween 40, 0.3% yeast extract and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0. In all experiments requiring induction of the *CUP1* promoter, 0.025 g/l CuSO₄ was added to YNO medium.

Plasmids

Plasmid YEpthio (Erdmann, 1989) contains a 4 kb *Bam*HI–*Xho*I fragment carrying *FOX3* inserted into YEp352 (Hill *et al.*, 1986). Plasmid YEpl05 (Ellison and Hochstrasser, 1991) contains the polyubiquitin gene *UBI4* fused behind a c-Myc epitope under the control of the inducible *CUP1* promoter (Butt *et al.*, 1984; Karin *et al.*, 1984). For our constructions the *Bam*HI–*Kpn*I CUP1mycUb cassette from YEpl05

Table II. Yeast and bacterial strains

Strain	Genotype	Source or reference
<i>S.cerevisiae</i>		
UTL-7A	<i>MATα ura3-52 trp1 leu2-3/112</i>	W.Duntze (Bochum)
XDC-10A	<i>MATα ura3-52 trp1 leu2-3/112</i>	W.Duntze (Bochum)
D273-10B	<i>MATα</i>	ATCC24657
JKR101	<i>MATα ura3-52 leu2-3/112 ade2 his4</i>	G.Schatz (Basel)
<i>pas7-1</i>	<i>MATα pas7-1 ura3-52 trp1 leu2-3/112</i>	This study
<i>pas7-1/1</i>	<i>MATα pas7-1ura3-52 trp1 leu2-3/112</i>	This study
<i>pas7-2</i>	<i>MATα pas7-2 ura3-52 trp1 leu2-3/112</i>	D.Leisman (Bochum)
Δ <i>pas7</i>	<i>MATα pas7::LEU2 ura3-52 trp1</i>	This study
<i>fox3-2</i>	<i>MATα fox3-2 trp1 ade2</i>	R.Erdmann (Bochum)
<i>fox3-2A</i>	<i>MATα fox3-2 ura3-52 trp1 leu2-3/112 ade2</i>	This study
<i>E.coli</i>		
TG1	Δ (<i>lac pro</i>) <i>supE thi hsdD5 F' [traD36 pro⁺ lacI^q lacZΔM15]</i>	O.Pongs (Bochum)

was subcloned into the *Bam*HI and *Kpn*I-digested Bluescript vector SK⁺ (Stratagene, USA) resulting in plasmid SK/mycUb.

*Bcl*I and *Bgl*II sites were introduced at the 5' end of the *PAS7* coding region using the PCR and the following primers: primer1 (5'-ATATAT'GATCA'GATCTATGCTCAGATATCATATGCAA-3'; the *Bcl*I and *Bgl*II sites are bold) and universal sequencing primer (5'-ATTAACCCTCACTAAA-3'). The *Sau*3A clone pRSP7/1.2 (see section DNA sequencing) containing *PAS7* with 53 bp of the 5' and 81 bp of the 3' non-coding region, respectively was used as template in the PCR. The fragment obtained by this PCR was digested with *Bcl*I and *Kpn*I, and cloned into *Bgl*II and *Kpn*I digested SK/mycUb by replacing the ubiquitin gene and resulting in SK/mycP7. Plasmid SK/mycP7 was digested with *Xba*I and *Kpn*I to obtain the CUP1mycPAS7 cassette which was cloned into *Xba*I and *Kpn*I digested vectors pRS316 (Sikorski and Hieter, 1989) and YEp352 (Hill *et al.*, 1986) resulting in plasmids pRSmycP7 and YEpmycP7, respectively. The amino-terminal amino acid sequence of the fusion protein is as follows: MCEQKLI-SEEDLGMQIRSL-Pas7p.

Other plasmids are specified in the sections that describe the isolation of *PAS7* and the construction of a *pas7* null allele. Recombinant DNA techniques, including enzymatic modification of DNA, fragment purification, bacterial transformation and plasmid isolation were performed essentially as described by either Maniatis *et al.* (1982) or Ausubel *et al.* (1989).

Isolation of the *pas7-1* and *pas7-2* mutants

The mutant strains *pas7-1* and *pas7-2* were obtained after mutagenesis of UTL-7A cells using ethyl methanesulfonate (Sherman *et al.*, 1979). The screening protocol included replica plating on YNO agar plates, fractionation of yeast cells and EM as described by Erdmann *et al.* (1989). Genetic analysis was performed by standard yeast techniques (Ausubel *et al.*, 1989).

Analytical procedures

Acetyl-CoA acyltransferase (3-oxoacyl-CoA thiolase; EC 2.3.1.16), catalase (EC 1.11.1.6), 3-hydroxyacyl-CoA epimerase (EC 5.1.2.3), cytochrome *c* oxidase (EC 1.9.3.1) and fumarate hydratase (fumarase; EC 4.2.1.2) were assayed by established procedures (Moreno de la Garza *et al.*, 1985; Veenhuis *et al.*, 1987).

Electron microscopy and immunocytochemistry

Intact yeast cells were fixed with potassium permanganate and prepared for electron microscopy as described by Erdmann *et al.* (1989). Immunocytochemistry on ultrathin sections of Lowicryl-embedded cells with anti 3-oxoacyl-CoA thiolase antibodies was performed as described by Douma *et al.* (1985).

Fractionation of yeast lysates and purification of peroxisomes

Preparation and fractionation of yeast lysates by differential centrifugation was performed as described (Erdmann *et al.*, 1989). For further subfractionation by isopycnic sucrose density gradient centrifugation, cell lysates of wild-type and mutant strains were loaded onto continuous 20–53% sucrose density gradients (24 ml volume). Centrifugation, fractionation of the gradient and preparation of samples for SDS–PAGE were carried out as described by Höhfeld *et al.* (1991).

Organellar pellets of oleate-induced wild-type and mutant strains were prepared according to Erdmann *et al.* (1989). Purified peroxisomes were obtained by loading the organelle pellets onto continuous 15–36% Nycodenz gradients (24 ml volume) with a cushion of 1 ml 42% Nycodenz dissolved in 5 mM MES (pH 6.0), 1 mM EDTA, 1 mM KCl, 0.1% ethanol and 8.5% sucrose. Centrifugation was performed for 2 h at 48 000 g in a Sorvall SS 90 vertical rotor at 4°C.

Western blotting

Samples were electrophoresed and electroblotted onto nitrocellulose according to standard protocols (Harlow and Lane, 1988). The nitrocellulose filters were incubated in the presence of anti-thiolase (dilution 1:5000), anti-SKL (1:200) or anti-Myc antibodies (1:2000) for 2 h at 30°C or overnight at 20°C. After washing with TBS–Tween 20 (0.05% Tween 20 in Tris-buffered saline) the filters were incubated with anti-rabbit IgG coupled alkaline phosphatase or anti-mouse IgG coupled horseradish peroxidase as second antibody. Protein–antibody complexes were visualized by treatment with colour or chemiluminescence developing reagents (Blake *et al.*, 1984; ECL system, Amersham).

Cloning and analysis of the *PAS7* gene

PAS7 was cloned by functional complementation of the *pas7-1* mutant with a yeast genomic library of *S.cerevisiae* contained in the *E. coli*–yeast shuttle vector YEp13 (Broach *et al.*, 1979). Transformation was carried out by a modified lithium acetate method (Gietz and Sugino, 1988). Leu⁺ transformants were replica-plated on YNO agar plates. Among 20 000 transformants, two had regained the ability to utilize oleic acid as the sole carbon source. The complementing plasmids were isolated as described by Ausubel *et al.* (1989). As judged by restriction analysis, both transformants contained the same 10 kb plasmid designated YEpP7/10.0. Subclones of YEpP7/10.0 were generated by introducing defined restriction fragments and fragments obtained through partial digestion of the genomic insert with *Sau*3AI into the *CEN4*–*URA3* vector pRS316 (Sikorski and Hieter, 1989). The resulting plasmids were tested for complementation by transformation of the *pas7-1* mutant, selection for Ura⁺ and subsequent screening on YNO agar plates.

DNA sequencing

To sequence the 1.26 kb *Sau*3AI fragment of plasmid pRSP7/1.2 (Figure 4), defined restriction fragments and deletion fragments generated by *Bal*31 exonuclease were subcloned into Bluescript vectors (Stratagene, USA). Single-stranded sequencing templates were prepared from transformed TG1 bacterial cells infected with M13R408 as described (Stratagene Cloning Systems). Sequence analysis was carried out using the dideoxy chain termination method (Sanger *et al.*, 1977). The inferred *PAS7* protein sequence was used to search EMBL Nucleotide Sequence Database (release 38) for similarities with other known protein sequences using the GENEPRO-program (Riverside Scientific Enterprise, Seattle, WA). Hydropathy analysis was carried out according to Kyte and Doolittle (1982).

Construction of a *pas7* null allele

The *PAS7* gene deletion construct (pJGD7) was made by first subcloning a 1.8 kb *Clai*I–*Eco*RV fragment and a 0.8 kb *Hinc*II–*Bam*HI fragment of the *PAS7* 5' and 3' non-coding region into Bluescript vectors in order to generate appropriate restriction sites and subsequently introducing

them into vector pJJ283 (Jones and Prakash, 1990) flanking the *LEU2* gene. Plasmid pJJGD7 was cut with *SacI* and the resulting fragment (one *SacI* site originates from the *Clal*-*EcoRV* fragment, the other from vector pJJ283) was introduced into wild-type strain UTL-7A. The resultant leucine-prototrophic transformant was mated with wild-type JKR101, the diploid was induced to sporulate and the meiotic progeny were examined by standard tetrad analysis. Southern analysis was performed as follows. Genomic DNA was prepared from the parent and transformant and digested with *BamHI*. The fragments were separated by electrophoresis on a 0.8% agarose gel, blotted to a nitrocellulose membrane and probed with a ³²P-labelled 4 kb *Clal*-*XbaI* fragment containing *PAS7* (Figure 7A).

Acknowledgements

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