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The import receptor for the peroxisomal targeting signal 2 (PTS2) in *Saccharomyces cerevisiae* is encoded by the PAS7 gene

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The import of peroxisomal matrix proteins is dependent on one of two targeting signals, PTS1 and PTS2. We demonstrate in vivo that not only the import of thiolase but also that of a chimeric protein consisting of the thiolase PTS2 (amino acids 1–18) fused to the bacterial protein β-lactamase is Pas7p dependent. In addition, using a combination of several independent approaches (two-hybrid system, co-immunoprecipitation, affinity chromatography and high copy suppression), we show that Pas7p specifically interacts with thiolase in vivo and in vitro. For this interaction, the N-terminal PTS2 of thiolase is both necessary and sufficient. The specific binding of Pas7p to thiolase does not require peroxisomes. Pas7p recognizes the PTS2 of thiolase even when this otherwise N-terminal targeting signal is fused to the C-terminus of other proteins, i.e. the activation domain of Gal4p or GST. These results demonstrate that Pas7p is the targeting signal-specific receptor of thiolase in *Saccharomyces cerevisiae* and, moreover, are consistent with the view that Pas7p is the general receptor of the PTS2. Our observation that Pas7p also interacts with the human peroxisomal thiolase suggests that in the human peroxisomal disorders characterized by an import defect for PTS2 proteins (classical rhizomelic chondrodysplasia punctata), a functional homologue of Pas7p may be impaired.

Keywords: 3-oxoacyl-CoA thiolase/peroxisomal targeting signal/peroxisome/receptor/Saccharomyces cerevisiae

Introduction

The function of most proteins is dependent on their correct subcellular localization. Their transport to their sites of action involves the specific recognition of targeting signals within the primary structure of the proteins. These short amino acid sequences are recognized by specific receptors, which then initiate a series of steps leading to protein translocation through membranes. Only a few such receptors have been identified so far.

In the case of peroxisomes, an essential metabolic compartment in eukaryotic cells, two different, evolutionarily conserved peroxisomal targeting signals (PTS1 and PTS2) are known to direct proteins into the matrix of these organelles. PTS1 is found at the extreme carboxy-terminus of many peroxisomal proteins and comprises the tripeptide SKL or variants thereof (for review, see Subramani, 1993). This tripeptide was identified initially in firefly luciferase (Gould et al., 1987) and is necessary and sufficient to target proteins to peroxisomes in diverse organisms, such as mammals, plants, insects and yeasts (Gould et al., 1990; Keller et al., 1991). Another subset of peroxisomal proteins possesses the amino-terminal PTS2. Sequence comparison of these proteins revealed the consensus RLX3HL within the first 30 amino acids (de Hoop and Ab, 1992; Rehling et al., 1996). The amino-terminus containing this consensus is necessary and sufficient to direct otherwise cytosolic proteins to the peroxisomal matrix. This has been shown for peroxisomal thiolase of rat (Tsukamoto et al., 1994) and *Saccharomyces cerevisiae* (Erdmann, 1994; Glover et al., 1994a) as well as for glyoxysomal malate dehydrogenase of watermelon (van der Klei et al., 1993). In addition, site-specific mutations in the PTS2 consensus impair peroxisomal import (Glover et al., 1994a; Gietl et al., 1994).

The elucidation of the molecular mechanisms underlying these peroxisomal sorting processes is severely hampered by the lack of an efficient in vitro import system based on purified peroxisomes. Therefore, we started a combined genetic and biochemical approach in *S.cerevisiae* to identify the essential components for protein import. This approach has led to the identification of 15 pas (peroxisome assembly deficient) complementation groups by us and others (Erdmann et al., 1989; van der Leij et al., 1992; Elgersma et al., 1993). Although the corresponding 15 PAS genes have been cloned, distinct functions have been proposed thus far only for the gene products of *PAS7* and *PAS10*. Among the pas mutants of *S.cerevisiae*, pas7 and pas10 are unique in showing only partial import deficiencies. In pas10 cells, proteins with the PTS1 signal remain cytosolic while thiolase, a PTS2 protein, is imported correctly (van der Leij et al., 1993). In contrast, pas7 cells show the opposite defect: thiolase is not imported but all PTS1 proteins are directed correctly to the peroxisomal matrix (Marzioch et al., 1994; Zhang and Lazarow, 1995). In humans, the failure to import polypeptides into peroxisomes has lethal consequences (Lazarow and Moser, 1995). Recently, the same partial import defects of either PTS1 or PTS2 proteins have been described for two out of 10 complementation groups in human fibroblast cell lines of patients suffering from peroxisomal diseases (Motley et al., 1994; Slaweccki et al., 1995). These diseases are the result of mutations affecting peroxisomal structure and/or function. These partial import defects in yeast and humans suggested the existence of two independent import receptors, one that recognizes the PTS1 and a second one that binds to the PTS2.

The *PAS10* gene of *S.cerevisiae* (van der Leij et al., 1994)
1993) and its homologues in two methylotrophic yeasts, \textit{PAS}8 of \textit{Pichia pastoris} (McCollum et al., 1993) and \textit{PER3/PAH2} of \textit{Hansenula polymorpha} (Nuttley et al., 1995; van der Klei et al., 1995), as well as its human counterpart (Dodd et al., 1995; Fransen et al., 1995; Wiemer et al., 1995), have been cloned. The \textit{P. pastoris} \textit{Pas8} protein and its human homologue were shown to bind peptides with SKL at the carboxy-terminus preferentially over the same peptides without SKL (Dodd et al., 1995; Fransen et al., 1995; Wiemer et al., 1995). The human gene can functionally rescue fibroblast cell lines having a PTS1 protein import deficiency (Dodd et al., 1995; Wiemer et al., 1995). These results were taken as evidence that these genes encode the PTS1 receptor. This interpretation was supported by studies using the two-hybrid system which indicated that Pas10p of \textit{S. cerevisiae} interacts with luciferase in a SKL-dependent manner (Brocard et al., 1994). In addition, Fransen et al. (1995) cloned the putative human PTS1 receptor by a two-hybrid screening of a human liver cDNA library with the 70 C-terminal amino acids of rat palmitoyl-CoA oxidase as bait. Moreover, recently it was shown that a PTS1-containing peptide can be cross-linked to Pas8p of \textit{P. pastoris} and that purified Pas8p binds this peptide with high affinity (nanomolar dissociation constant) (Terlecky et al., 1995). Together, these accumulated data indicate that \textit{Pas10} of \textit{S. cerevisiae} and its homologues encode the PTS1 receptor.

In contrast to the PTS1 import deficiencies which have been found in mutants of three different yeast species, mutants displaying a PTS2 deficiency have only been isolated from \textit{S. cerevisiae}. PTS2-deficient mutants belong to the \textit{pas7} (\textit{pebl}) complementation group (van der Leij et al., 1992; Marzioch et al., 1994; Zhang and Lazarow, 1995).

We have described previously the cloning of the \textit{S. cerevisiae} \textit{Pas7} gene and the partial characterization of its gene product Pas7p (Marzioch et al., 1994). The majority of the Pas7 protein was recovered in the cytosolic fraction, only a minor portion segregated in density gradients with peroxisomes and then only in cells which possess thiolase.

Based on these results, we proposed that Pas7p binds thiolase (Fox3p) in the cytosol and might shuttle between the cytosol and the peroxisomal compartment in performing its function as the putative PTS2 receptor. In contrast, Zhang and Lazarow (1995) who also reported the cloning and sequencing of the same gene, which they termed \textit{PEB1}, found Pas7p/Peb1p in the peroxisomal matrix. They discussed a chaperone (Hsp70)-like function for this protein.

In accordance with our shuttle model, we report here the first evidence that Pas7p binds thiolase both \textit{in vivo} and \textit{in vitro} and that this interaction is PTS2 dependent. Thus, Pas7p possesses the characteristics expected of a PTS2 receptor.

**Results**

\textbf{Import of a PTS2-\beta-lactamase fusion protein into peroxisomes requires Pas7p}

It had been shown that peroxisomal import of thiolase in \textit{S. cerevisiae} is strictly dependent on Pas7p (Marzioch et al., 1994). We wanted to investigate whether this holds true also for a chimeric protein consisting of the PTS2 of thiolase fused to a reporter protein. For that purpose, the amino-terminal 18 amino acids of yeast thiolase, which are necessary and sufficient for peroxisomal targeting of this protein (Erdmann, 1994; Glover et al., 1994a), were fused to \beta-lactamase lacking its own amino-terminal targeting signal required for secretion from bacteria. This reporter protein has been shown to be cytosolic in yeast in the absence of added targeting information (data not shown). Moreover, this bacterial protein has been used successfully for targeting studies in the methylotrophic yeast \textit{H. polymorpha} (Waterham et al., 1994; Faber et al., 1995). The PTS2-\beta-lactamase fusion protein was placed under control of the thiolase/\textit{FOX3} promoter (Einerhand et al., 1991) in the multicopy vector pEMBL\textit{yex4} (pEMBL/PTS2-\beta-lac) and transformed in wild-type and \textit{Pas7} cells.

In both cases, the chimeric protein could be detected in crude extracts of the transformants with a specific anti-\beta-lactamase antibody (data not shown), confirming high expression of the fusion protein. To determine the subcellular localization of PTS2-\beta-lactamase in the transformants, immunocytochemical experiments using antibodies against \beta-lactamase were performed. In the two transformants there was a clear difference with respect to the immunogold labelling of peroxisomes. Although the cytosol and nuclei of both transformants showed an intensive immunogold labelling (due to the overexpression of the protein from a multicopy vector) there was, however, a clear difference with respect to the labelling of peroxisomes. While peroxisomes of wild-type cells (Figure 1B) were densely labelled, those of \textit{Pas7} cells contained no gold particles (Figure 1A). Since the background of the two strains used only differs in the fact that \textit{Pas7} cells lack the Pas7 protein, our results indicate that functional Pas7p is essential not only for import of thiolase but also for lactamase fused to the first 18 amino acids of thiolase. These essential 18 amino-terminal residues contain the proposed PTS2 consensus.

This conclusion was also confirmed by biochemical analysis. The PTS2-\beta-lactamase-encoding construct in the single copy vector YCp\textit{plac33} (YCp/PTS2-\beta-lac) was transformed in \textit{Pas7} and \textit{fox3}-2A cells, the latter strain being deficient in thiolase protein. Cell homogenates of the transformants were fractionated by sucrose density centrifugation and the gradient fractions subsequently analysed by Western blot analysis. As shown in Figure 2B, a significant amount of the fusion protein co-migrated with the peroxisomal marker catalase and also tails into less dense fractions which most likely contain 'lighter' peroxisomal structures (Wiebel and Kunau, 1992). How-

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**Fig. 1.** Immunocytochemical detection of PTS2-\beta-lactamase in oleate-induced \textit{Pas7} cells (A) and wild-type cells (B) using specific antibodies against \beta-lactamase. Both strains were transformed with pEMBL/PTS2-\beta-lac. The fusion protein is not imported into peroxisomes of \textit{Pas7} cells but can be detected in wild-type peroxisomes. M, mitochondrion; N, nucleus; P, peroxisome; bars represent 1 \mu m.
These results indicate that the proper targeting of the PTS2-containing hybrid protein is dependent on Pas7p function and thus underlines the specificity of the Pas7 protein for the PTS2 sequence.

**Thiolase binds to Pas7p in vivo**
In order to investigate whether the Pas7 protein binds thiolase (Fox3p) *in vivo*, the GAL4-based two-hybrid system was employed as a direct assay of protein–protein interaction (Fields and Song, 1989; Chien et al., 1991). PAS7, FOX3 and FOX3 lacking the first 48 nucleotides of its open reading frame were cloned in-frame downstream of either the GAL4 DNA binding or activation domain (see Materials and methods). These plasmids, together with constructs detailed below, were co-expressed in different pairwise combinations in the *S. cerevisiae* host strain PCY2 (Chevray and Nathans, 1992). Whole cell extracts of the transformants were assayed for β-galactosidase activity (Table IA). The results showed that the combination of Pas7p fused to the DNA binding domain of Gal4 (DB-Pas7p) and Fox3p fused to the activation domain of Gal4 (AD-Fox3p) confers a striking stimulation of β-galactosidase activity compared with either fusion protein alone (Table IA). In contrast, no interaction was found between DB-Pas7p and AD-APTS2-Fox3p. Identical results were obtained by expression of the fusion proteins in the yeast reporter strain HF7c (Clontech) where activation of the HIS3 reporter gene could be achieved (Figure 3).

Surprisingly, Pas7p fused to the activating rather than the binding domain of Gal4 (AD-Pas7p) is not capable of interacting with thiolase fused to the DNA binding domain of Gal4 (DB-Fox3p) (Table IA, Figure 3). The fact that both the truncated and the full-length thiolase are enzymatically active when fused to either Gal4 domain (Table IA) indicates that these proteins are properly folded. Therefore, the inability of AD-Pas7p to bind thiolase might result from an interference of Gal4-AD with the thiolase binding either by blocking the thiolase binding site on Pas7p or by causing incorrect folding of Pas7p.

Using the same approach, we investigated whether Pas7p was capable of binding to the human peroxisomal thiolase (Bout et al., 1988). Co-transformation with DB-Pas7p and AD-human thiolase into the HF7c reporter strain resulted in activation of both the LacZ and HIS3 reporter genes (Figure 3).

Taken together, these data provide strong evidence that Pas7p binds thiolase *in vivo*, provided that the thiolase PTS2 is intact. Furthermore, as the chimeric proteins of full-length Fox3p or APTS2-Fox3p were both enzymatically active (Table I), this seems to indicate that Pas7p recognizes the dimeric, active form of thiolase.

**Thiolase associates with Pas7p in vitro**
As an independent confirmation that Pas7p and thiolase specifically interact, the ability of Pas7p to precipitate thiolase from a crude extract was tested. Cells of the wild-type strain UTL-7A and Δpas7 were transformed with the multicopy plasmid YEpmycP7 (Marziocchi et al., 1994). This plasmid encodes the full-length Pas7p with a myc epitope tag at its amino-terminus under the control of the CUP1-promotor. The transformants were grown and crude extracts prepared (see Materials and methods). Immuno-

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Fig. 2. Subcellular location of PTS2-β-lactamase in oleate-induced Δpas7 cells (A) and fox3-2A cells (B) that were transformed with YCP/PTS2-β-lac. Cell lysates were subjected to fractionation on a 20-55% sucrose density gradient. Activities of mitochondrial (fumarase) and peroxisomal (thiolase and catalase) marker enzymes indicate the localization of the organelles. Equal amounts of each fraction (0.1%) were immunologically analysed for the presence of PTS2-β-lactamase using specific antibodies against β-lactamase.

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however, as the protein content of fractions 4–10 was three times lower than that of fractions 12–18 (data not shown), and considering the more intensive signal of the former fractions compared with the latter ones (Figure 2B), more of the fusion protein accumulated in wild-type peroxisomes than in the intermediate peroxisomal structures. In addition, a major part of the fusion protein was detected on top of the gradient in fox3-2A cells, indicating a cytosolic location. This observation cannot be explained solely by the partial disruption of peroxisomes during cell fractionation since the ratio of peroxisomal to cytosolic PTS2-β-lactamase was smaller than that of catalase. We conclude that although the first 18 amino acids of thiolase are sufficient to direct the β-lactamase fusion protein to peroxisomes, this targeting is less efficient than that of wild-type thiolase. Most importantly, when expressed in Δpas7 cells, PTS2-β-lactamase remained completely cytosolic and was not found in either wild-type or intermediate structures, demonstrating that the targeting of the reporter protein to peroxisomes is impaired (Figure 2A).
precipitation of the myc-tagged Pas7p was performed by the use of monoclonal α-myc antibodies (9E10) bound to α-mouse IgG magnetic beads (Dynal). After washing of immunocomplexes, bound proteins were eluted from the beads with Laemmli sample buffer and subjected to denaturing electrophoresis and blotting.

While no α-myc-precipitable proteins were isolated from extracts of untransformed strains (Figure 4A, lanes 1 and 3), the precipitates obtained from the transformants showed a 45 kDa protein band on Coomassie-stained SDS–PAGE (Figure 4A, lanes 2 and 4). The 53 and 30 kDa polypeptides observed in all lanes correspond to the heavy and light chain of the monoclonal α-myc and α-mouse antibodies; the appearance of the additional 50 and 80 kDa proteins is due to the α-mouse IgG antibodies (data not shown). α-Myc (Figure 4B, lanes 2 and 4) and α-Pas7p antibodies (Figure 4C, lanes 2 and 4) as well as α-thiolase antibodies (Figure 4D) immunodecorated a band of ~45 kDa in immunoprecipitates of transformed cells. This band was not observed in the controls (untransformed strains). Since the calculated molecular mass of mycPas7p (44.4 kDa) and thiolase (44.7 kDa) is almost identical, we conclude that both proteins co-immunoprecipitated as the 45 kDa band. These data confirm that Pas7p interacts tightly and specifically with thiolase in solution. Interaction of Pas7p and thiolase does not require functional peroxisomes.

In order to investigate whether the Pas7p–thiolase interaction requires import-competent peroxisomes, co-immunoprecipitation of these proteins was tested in type 1 pas mutants (Höhnfeld et al., 1992). These mutants contain no morphologically detectable peroxisomes. This experiment was performed with fox3-2A, Δpas2 and Δapas6 cells that had been transformed with plasmid YEpmycP7. In all immunoprecipitates the 45 kDa band was visible (Figure 5A, lanes 1, 2 and 3) and could be immuno-decorated by either α-myc or α-Pas7p antibodies. When the fox3-2A mutant defective in the gene for thiolase (Marzioch et al., 1994) was transformed and used as a source of cell extract no Fox3p co-immunoprecipitated with mycPas7p (Figure 5D, lane 1). This result demonstrates the specificity of the antibodies used and shows that the 45 kDa band detected by α-thiolase antibodies (Figures 4D and 5D) is indeed the Fox3 polypeptide. The Δpas2 mutant is biochemically characterized by residual peroxisomal membrane structures that fail to import matrix enzymes (Wiebel and Kunau, 1992) in contrast to Δapas6 mutant cells in which no peroxisomes or peroxisomal membrane structures have been detected (Kunau et al., 1993). Figure 5 shows that in both Δpas2 and Δapas6 cells carrying the mycPas7 fusion construct, mycPas7p (Figure 5B and C) co-precipitates with thiolase (Figure 5D). These

### Table 1A. Analysis of Pas7p–thiolase interaction in a two-hybrid system

<table>
<thead>
<tr>
<th>Pas7p domain fused to:</th>
<th>GAL4 AD domain fused to:</th>
<th>Colony colour</th>
<th>β-Galactosidase activity (nkat/mg protein)</th>
<th>Thiolase activity (nkat/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pas7p</td>
<td>Fox3p</td>
<td>Blue</td>
<td>4659.3</td>
<td>11.8</td>
</tr>
<tr>
<td>Fox3p</td>
<td>Pas7p</td>
<td>White</td>
<td>14.7</td>
<td>12.8</td>
</tr>
<tr>
<td>Pas7p</td>
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<td>White</td>
<td>17.9</td>
<td>0.0</td>
</tr>
<tr>
<td>None</td>
<td>Fox3p</td>
<td>White</td>
<td>1.3</td>
<td>7.9</td>
</tr>
<tr>
<td>Pas7p</td>
<td>Fox3(D1–16 aa)p</td>
<td>White</td>
<td>0.3</td>
<td>6.4</td>
</tr>
<tr>
<td>Fox3(D1–16 aa)p</td>
<td>Pas7p</td>
<td>White</td>
<td>0.4</td>
<td>10.4</td>
</tr>
<tr>
<td>None</td>
<td>Fox3(D1–16 aa)p</td>
<td>White</td>
<td>0.9</td>
<td>4.2</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>White</td>
<td>0.9</td>
<td>0.0</td>
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</table>

### Table 1B. Analysis of Pas7p–PTS2 interaction in a two-hybrid system

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<tr>
<th>Pas7p domain fused to:</th>
<th>GAL4 AD domain fused to:</th>
<th>Colony colour</th>
<th>β-Galactosidase activity (nkat/mg protein)</th>
<th>β-Lactamase activity (nkat/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pas7p</td>
<td>PTS2–β-lactamase</td>
<td>Blue</td>
<td>3725.8</td>
<td>119.5</td>
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<tr>
<td>PTS2–β-lactamase</td>
<td>Pas7p</td>
<td>White</td>
<td>54.0</td>
<td>104.1</td>
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<tr>
<td>Pas7p</td>
<td>β-Lactamase</td>
<td>White</td>
<td>62.7</td>
<td>104.6</td>
</tr>
<tr>
<td>β-Lactamase</td>
<td>Pas7p</td>
<td>White</td>
<td>50.9</td>
<td>100.1</td>
</tr>
<tr>
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<td>PTS2–β-lactamase</td>
<td>White</td>
<td>39.8</td>
<td>120.0</td>
</tr>
<tr>
<td>None</td>
<td>β-Lactamase</td>
<td>White</td>
<td>26.7</td>
<td>155.0</td>
</tr>
</tbody>
</table>

Extracts were made from double transformants expressing various plasmid combinations and assayed for β-galactosidase, β-lactamase and thiolase activity. Untransformed PCY2 cells showed a β-galactosidase activity of 1.1 nkat/mg protein and no measurable thiolase or β-lactamase activity. PCY2 cells transformed with pCLI (Fields and Song, 1989) showed no measurable thiolase or β-lactamase activity but a β-galactosidase activity of 147 541 nkat/mg protein.
data demonstrate that Pas7p and thiolase associate even in the absence of a functional peroxisomal import machinery and, furthermore, that no peroxisomal membrane is necessary for this interaction.

**Pas7p binds to the PTS2**

To investigate the possibility that Pas7p binds directly to the PTS2 targeting signal, the PTS2–β-lactamase fusion protein described above was tested in the two-hybrid system. Double transformants of strain PCY2 with DB-Pas7p and either the PTS2–β-lactamase fusion (AD-PTS2–β-lactamase) or β-lactamase (AD-β-lactamase) were assayed for β-galactosidase and β-lactamase activity (Table IB). The data clearly show that Pas7p, in combination with the PTS2–β-lactamase fusion protein was able to activate the transcription of the reporter gene *LacZ*, whereas no activation was observed when Pas7p and native β-lactamase were co-expressed. These results demonstrate that Pas7p binds specifically to the first 18 amino acids of thiolase comprising the PTS2. Since the Gal4–β-lactamase fusion can be shown to possess β-lactamase activity, we conclude that this interaction occurs between correctly folded proteins.

**PAS7 is an extragenic high copy suppressor for a temperature-sensitive mutant PTS2**

To investigate further the possible interaction of Pas7p with the PTS2 of thiolase, we took advantage of a mutant...
The PTS2 confirms further can be the point observation shows that 2A and transform the FOX3 2A and construct wild-type temperature of prohibited at cells. The to similar mentioning the temperature-sensitive gene arginine cells (F.Niesen and Fox3p, PTS2 of phenotype temperature-sensitive growth Overexpression 6. Fig. P.Rehling et fusion protein binds 2906

Fig. 6. Functional complementation of the fox3-2A mutant in terms of growth on oleic acid medium at 32 and 23°C. Growth behaviour of different fox3-2A transforms is compared with wild-type. Overexpression of Pas7p (pPR7/4) leads to suppression of a temperature-sensitive phenotype caused by a mutation (R4G) in the PTS2 of thiolase (pFN3/8). Construct pWS-2, expressing unmodified Fox3p, can complement fox3-2A at 32°C as well as 23°C. fox3-2A cells transformed with pFN3/8 alone are unable to complement the mutant at 32°C.

FOX3 gene recently described by Glover et al. (1994a) and independently constructed and analysed in our group (F.Niesen and W.-H.Kunau, unpublished). This FOX3 allele is mutated in the PTS2-encoding region so that the arginine at position 4 of Fox3p is replaced by glycine (R4G). This arginine residue is one of the few amino acids within the PTS2 consensus that is strongly conserved between mammals, plants and yeasts (de Hoop and Ab, 1992; Rehling et al., 1996). The mutated allele confers a temperature-sensitive phenotype in terms of complementing the fox3-2A mutant when expressed in fox3-2A cells. The growth of transformants on oleic acid plates is prohibited at the restrictive temperature of 32°C but is similar to that of the wild-type strain at the permissive temperature of 23°C (Figure 6). In contrast, the analogous wild-type construct (pWS-2) was able to rescue the fox3-2A defect at both temperatures. It has been suggested that the R4G PTS2 mutation causes a destabilization of the interaction between factors that recognize the PTS2 and the PTS2 itself (Glover et al., 1994a). The temperature-sensitive FOX3 allele and PAS7 on multicopy plasmids (pFN3/8 and pPR7/4) respectively were used to co-transform the fox3-2A mutant. Transformants were analysed with regards to growth on oleate at the permissive and restrictive temperatures (Figure 6). In contrast to fox3-2A cells carrying the ts PTS2 allele alone, fox3-2A cells co-transformed with the PAS7 gene (pPR7/4) regained the ability to grow on oleic acid at 32°C (Figure 6). This observation shows that the temperature-sensitive effect of the point mutation R4G in the PTS2 consensus of thiolase can be suppressed by overexpression of Pas7p. This result further confirms a direct binding of the Pas7 protein to the PTS2 of thiolase.

A GST–PTS2 fusion protein binds to Pas7p in vitro

The PTS2 of *S.cerevisiae* thiolase was fused to the C-terminus of glutathione-S-transferase (GST). This fusion protein and GST alone were expressed in *Escherichia coli* and purified by affinity chromatography. mycPas7p was isolated by immunoprecipitation from fox3-2A cells. Aliquots of the Dynabeads to which mycPas7p was bound was washed and equal quantities subjected to SDS–PAGE and immunoblotting. Immunoblots were probed with antisera raised against a GST–Pas7p fusion protein that was able to recognize mycPas7p as well as GST. Immunoprecipitated mycPas7p incubated with GST–PTS2 (lane 1) or GST (lane 2).

**Fig. 7.** *In vitro* binding of mycPas7p to a GST–PTS2 fusion protein. The mycPas7p fusion protein was expressed in fox3-2A cells and immunoprecipitated from a crude extract using monoclonal α-myc antibodies bound to Dynabeads via α-mouse IgG. After washing, equal aliquots were incubated with the same amount of either GST or GST–PTS2 fusion protein that had been isolated from *E.coli*. Samples were washed and equal quantities subjected to SDS–PAGE and immunoblotting. Immunoblots were probed with antisera raised against a GST–Pas7p fusion protein that was able to recognize mycPas7p as well as GST. Immunoprecipitated mycPas7p incubated with GST–PTS2 (lane 1) or GST (lane 2).

**Fig. 7.** *In vitro* binding of mycPas7p to a GST–PTS2 fusion protein. The mycPas7p fusion protein was expressed in fox3-2A cells and immunoprecipitated from a crude extract using monoclonal α-myc antibodies bound to Dynabeads via α-mouse IgG. After washing, equal aliquots were incubated with the same amount of either GST or GST–PTS2 fusion protein that had been isolated from *E.coli*. Samples were washed and equal quantities subjected to SDS–PAGE and immunoblotting. Immunoblots were probed with antisera raised against a GST–Pas7p fusion protein that was able to recognize mycPas7p as well as GST. Immunoprecipitated mycPas7p incubated with GST–PTS2 (lane 1) or GST (lane 2).

**Pas10p, the PTS1 receptor and Pas7p interact in a two-hybrid system**

It has been reported that tetratricopeptide repeat (TPR) proteins (Lamb et al., 1995) and WD-40 proteins (Neer et al., 1994) are functionally related (Goebl and Yanagida, 1991; van der Voorn and Ploegh, 1992). Thus, we wanted to know whether Pas7p, which is a member of the WD-40 family, and Pas10p, which belongs to the TPR family and is thought to be the *S.cerevisiae* PTS1 receptor, can physically interact. Using the GAL4 two-hybrid system, it could be shown that double transformants carrying plasmids encoding DB-Pas7p and AD-Pas10p showed transcriptional activation of the reporter genes HIS3 (Figure 8A) and *LacZ* (Figure 8B), suggesting that these two proteins do indeed associate. Furthermore, this inter-
action could also be demonstrated in vitro by affinity chromatography using recombinant proteins (to be published elsewhere). These results are surprising since the phenotypes of Δpas7 and Δpas10 clearly show that the import of PTS2 and PTS1 proteins in *S.cerevisiae* are independent of one another, Δpas7 mutants properly sorting PTS1 proteins but not PTS2 proteins, and Δpas10 mutants exhibiting the reciprocal effect.

**Pas7p is found in more than one subfraction of peroxisomes**

We previously reported that a myc-tagged Pas7 protein is found predominantly in the cytosol, while a minor part of it associates with peroxisomes (Marzioch *et al.*, 1994). Based on these results, we have proposed a model for Pas7p function suggesting that Pas7p might direct thiolase to peroxisomes by shuttling between cytosol and peroxisomal membranes. In order to test whether that portion of Pas7p co-migrating with peroxisomes in sucrose gradients might be physically associated with the peroxisomal membranes, we subfractionated peroxisomes (together with mitochondria) by means of a pH 8 step and carbonate extraction. An organelle pellet, prepared from Δpas7 cells expressing myc-tagged Pas7p from a multicopy vector, was treated with 10 mM Tris, pH 8.0. This step has been shown to release peroxisomal matrix proteins efficiently (McCammon *et al.*, 1990). A subsequent centrifugation at 200 000 g separated soluble from membrane-bound proteins. Incubation of the pellet in 100 mM Na₂CO₃ solution, pH 11.5, followed by a second centrifugation step (200 000 g) is thought to separate peripheral and integral membrane proteins. Surprisingly, immunoblot analysis demonstrates that the 44.5 kDa mycPas7p fusion protein was detectable in all three fractions obtained (Figure 8): it was extractable with the Tris buffer pH 8.0 and was also found in the supernatant and pellet fractions obtained after treatment with Na₂CO₃ buffer. The complete separation of the peroxisomal matrix protein thiolase and the integral membrane protein Pas3p into the expected fractions served as internal controls. The higher molecular weight protein detected in the Na₂CO₃ supernatant by using α-Pas3p antibodies is due to their cross-reaction with a mitochondrial protein (Th.Krause and W.-H.Kunau, in preparation). These results confirm our previous finding that a portion of the cellular Pas7 protein is associated with peroxisomes. In addition, they indicate that most of the peroxisomal Pas7p is membrane bound. Zhang and Lazarow (1995) have reported recently that Peb1p (Pas7p) is an intra-peroxisomal protein. The reason for this obvious discrepancy is unclear; however, it is important to note that Zhang and Lazarow based their conclusion on data obtained by different techniques.

**Discussion**

Once targeting signals have been identified, it is logical to ask which components of the cellular protein sorting machinery recognize these signals. In contrast to the import of PTS1 proteins, the import of the PTS2 protein thiolase into peroxisomes of *S.cerevisiae* requires the gene product of PAS7 (Marzioch *et al.*, 1994; Zhang and Lazarow, 1995). Based on genetic and biochemical data, we proposed previously that Pas7p might act as an import receptor by targeting thiolase from the cytosol to the peroxisomal membrane (Marzioch *et al.*, 1994). Our proposal suggested that Pas7p might have a dual function.
in binding the targeting signal of thiolase and in passing the protein on to a peroxisome-bound docking protein. This study provides the first in vivo and in vitro experimental evidence that the PTS2 of thiolase is indeed specifically recognized by Pas7p.

Several independent lines of evidence show that the binding of thiolase to Pas7p: (i) does not require a peroxisomal membrane; (ii) is PTS2-dependent; (iii) does not need a free amino-terminus close to the PTS2; and (iv) seems to occur with folded thiolase. By analogy to protein import into mitochondria and chloroplasts, it was expected that a peroxisomal import receptor would be a membrane protein located at the outer surface of the peroxisome. Pas7p, however, is a very hydrophilic protein. Furthermore, two conflicting localizations have been reported for Pas7p. Marzioch et al. (1994) reported a primarily cytosolic location whereas Zhang and Lazarow (1995) found it exclusively in the peroxisomal matrix. Although these conflicting results greatly influence the conclusions about the biological role which Pas7p might play in thiolase import (see below), they are both in agreement with our observation that the binding event between thiolase and Pas7p does not require the peroxisomal membrane.

The first step of the shuttle mechanism proposed by us (Marzioch et al., 1994) predicted not only that Pas7p and thiolase interact without the participation of the peroxisomal membrane but, in addition, do so in a PTS2-dependent manner. Indeed, that is exactly what is demonstrated by the three different in vivo experiments, an import experiment using a PTS2–β-lactamase fusion protein (Figures 1 and 2), the two-hybrid system (Table 1) and the observation that overexpression of PAS7 from a multicopy vector suppressed the ts phenotype of a mutant allele of the PTS2 (Figure 6).

The co-immunoprecipitation (Figures 4 and 5) and in vitro binding data (Figure 7) argue against a merely transient interaction between Pas7p and the PTS2 and suggest a tight binding such as might be required for Pas7p to shuttle the PTS2 protein through the cytosol to the peroxisomal membrane. However, this interpretation also requires a mechanism for releasing the PTS2 protein from Pas7p when it has reached the peroxisome. It is conceivable that this is achieved by a conformational change of Pas7p caused by binding to a putative docking protein.

The finding that a targeting signal is recognized by a cytosolic protein which subsequently participates in a docking event at the membrane to be passed through is not without precedent. A number of similar events have been described, among them SRP binding to signal sequences of eukaryotic proteins targeted for secretion (Kurzchalia et al., 1986). Sec B/Sec A interacting with prokaryotic signal sequences of proteins to be excreted (Randall and Hardy, 1995; Watanabe and Blobel, 1995), nuclear targeting receptors such as importin recognizing nuclear targeting sequences (Powers and Forbes, 1994) and mitochondrial import ‘factors’ binding to mitochondrial targeting sequences (Murakami and Mori, 1990; Hachiya et al., 1994).

A docking protein for the Pas7p–thiolase complex at the peroxisomal membrane has yet to be identified. The available genetic evidence, in the form of pas mutant phenotypes, does not favour the possibility that such a putative docking protein would be specific for the PTS2 import pathway. Only one complementation group with a partial import defect of PTS2 proteins exists among the human fibroblast cell lines from patients with peroxisomal disorders and among the yeast pas mutants. It is conceivable that a docking protein could serve for both the PTS1 and the PTS2 receptor pathways. In this case, all of the known PAS membrane proteins are candidates for such a function.

An especially important aspect of the two-hybrid experiments is the possibility that Pas7p binds thiolase in a folded state. This is suggested by the fact that the fusion protein of the Gal4 activating domain and thiolase showed thiolase activity. The recently established crystal structure of dimeric thiolase showed that the first 27 amino acids of both amino-termini protrude from the determined three-dimensional structure and should, therefore, be easily accessible (Mathieu et al., 1994). Moreover, the conclusion that Pas7p binds an active thiolase dimer helps to explain the recent, unexpected results of 'piggy-back experiments' (Glover et al., 1994b; McNew and Goodman, 1994). In these experiments, truncated thiolase molecules lacking the amino-terminal PTS2 sequence could be translocated into peroxisomes only in conjunction with wild-type PTS2 containing thiolase. Recognition and binding of the PTS2 signal on the wild-type member of a mixed thiolase dimer might be sufficient to initiate translocation of both subunits. The binding of oligomeric thiolase to Pas7p would not necessarily mean that oligomeric proteins are translocated through the peroxisomal membrane; however, in the light of the recent report that the attachment of a PST1 (SKL signal) can facilitate the transport of gold particles with diameters up to 9 nm into peroxisomes (Walton et al., 1995), one will have to take such a possibility into account. It is of course possible that the import of matrix proteins into peroxisomes involves an entirely different as yet unknown mechanism.

Our results clearly demonstrate that Pas7p specifically interacts with the PTS2 of thiolase. However, this does not exclude an additional interaction between Pas7p and the remaining major part of thiolase. This possibility is suggested by the observation that, in comparison with wild-type thiolase, PTS2–β-lactamase shows not only a weaker interaction with Pas7p in the two-hybrid system but is also less efficiently imported into peroxisomes in vivo. It is possible that the PTS2 is bound by Pas7p with high affinity and that the resulting complex is stabilized by additional low affinity binding sites between the proteins.

It is interesting to note that the amino-terminus of PTS2 proteins with their consensus within the first 20–30 amino acid residues can be extended by a large number of additional amino acid residues and still be recognized by Pas7p. This conclusion was suggested by the nature of the thiolase–Gal4 fusion protein which was used in this study. In this construct, thiolase was fused via its amino-terminus to the carboxy-terminus of the Gal4 activation domain. It had been reported that the PTS2 consensus of mammalian thiolase is not at the extreme amino-terminus and thus can be considered an internal targeting signal (Swinkels et al., 1991). Our results show that the PTS2 consensus can be moved much further away from the
amino-terminus and still function correctly. This suggests that if the PTS2 binds to a pocket within Pas7p, it might actually interact as a hairpin rather than a straight stretch of amino acids. However, the PTS2 could also form a surface-surface interaction with Pas7p, as has been shown for some protein-peptide interactions (Stanfield and Wilson, 1995).

Taken together, the findings presented here demonstrate that Pas7p has all the properties expected of a cytosolic PTS2 receptor. We have thus substantiated experimentally the first step of the proposed shuttle mechanism (Marzioch et al., 1994) in which Pas7p starts thiolase import by specific binding to the PTS2. The identity of the protein and/or lipid which functions as the predicted binding site for this complex at the peroxisomal membrane must now be determined.

Our results do not rigorously exclude a second proposed function of Pas7p. Zhang and Lazarow found Pas7p entirely inside peroxisomes and proposed that this protein may be an intraorganellar receptor for thiolase which, by analogy to the mitochondrial Hsp70 and BiP, pulls thiolase into the peroxisome and/or contributes to the folding of thiolase within the organelle (Zhang and Lazarow, 1995). However, this mode of action would require an as yet unknown extra-peroxisomal PTS2 receptor for which there is no evidence in any of the several extensive genetic screens which have been conducted in several laboratories. In addition, the construct used by Zhang and Lazarow (a triple HA tag fused to the C-terminus of Pas7p) might have resulted in an unphysiological localization. For example, if one assumes that Pas7p shuttles thiolase not only to but also through the peroxisomal membrane, one could envisage that the C-terminal HA tag might interfere with the exit of Pas7p. This would result in an entirely peroxisomal localization of Pas7p and, in addition, one would expect a somewhat less efficient growth on oleate due to cytosolic depletion. This is exactly what Zhang and Lazarow reported. In fact, our results of subperoxisomal fractionation of that part of Pas7p which is associated with peroxisomes suggested that peroxisomal Pas7p is partly membrane bound (peripheral and integral) and behaves partly as if it were soluble in the matrix. In addition, the PTS2-specific binding of thiolase by Pas7p argues against a chaperone-like function of the latter, since neither mitochondrial Hsp70 nor BiP specifically bind targeting sequences.

The PTS1 receptor has been identified in both yeast and humans. For these proteins, too, there are conflicting reports of intracellular localization, ranging from predominantly cytosolic (Dodd et al., 1995; Wiemer et al., 1995) to entirely membrane bound (Fransen et al., 1995; Terlecky et al., 1995). The shuttle mechanism first proposed by us for Pas7p has also been adopted to explain the role of the PTS1 receptor in H. polymorpha (van der Klei et al., 1995) and humans (Dodd et al., 1995; Wiemer et al., 1995).

Our finding that Pas7p interacts with Pas10p, the PTS1 receptor of S. cerevisiae, in the two-hybrid system is particularly interesting. Although genetic evidence clearly demonstrates that the two PTS-specific import pathways in S. cerevisiae are independent of each other (van der Leij et al., 1993; Marzioch et al., 1994; Zhang and Lazarow, 1995), this observation may indicate that the import complex docking to the peroxisomal membrane contains more components than previously anticipated. In this respect, it is important to note that two patients with mutations in the PTS1 receptor exhibit different phenotypes. One patient has a partial import defect restricted to PTS1 proteins while the other one mislocalizes both PTS1 and PTS2 proteins (Dodd et al., 1995; Wiemer et al., 1995). The physiological significance of our findings in the two-hybrid system is currently being investigated.

The fact that Pas7p recognizes not only the PTS2 of yeast thiolase but also human thiolase suggests conservation of the peroxisomal protein import machinery between yeast and human. It supports the hypothesis that not only peroxisomal targeting signals but also the receptor machinery may be conserved between lower and higher eukaryotes. Moreover, it provides a molecular explanation for the same partial import deficiencies for PTS1 and PTS2 proteins found among peroxisomal yeast mutants and fibroblast cell lines from patients with peroxisomal disorders (Motley et al., 1994; Slawecki et al., 1995). On the basis of the present results and in view of the fact that the PTS2 consensus is highly conserved among PTS2 proteins (de Hoop and Ab, 1992; Rehling et al., 1996), it seems very likely that Pas7p homologues will exist in other species of yeast and higher eukaryotes and will function there as the general PTS2 receptor.

Materials and methods

Strains and general methods

Genotypes of yeast strains used in this study are as follows: UTL1-7A is MA1a, ura3-52, leu2-3/112, trpl1 (W.Dunztch, Bochum); Aps2 is MA1a, pas2::LEU2, ura3-52, trpl1 (Wiebel and Kunau, 1993); Aps6 is MA1a, pas6::LEU2, ura3-52, trpl1 (Erdmann and Kunau, 1994); Aps7 is MA1a, pas7::LEU2, ura3-52, trpl1 (Marzioch et al., 1994); fox3-2A is MA1a, fox3-2, ura3-52, leu2-3/112, trpl1, ade2 (Marzioch et al., 1994); FCY2 is MA1a, Dgal4, Aagl80, URA3::GAL1-lacz, lys2-80, URA3::GAL1-lacz (Zhang, 1995).

This would result in an entirely peroxisomal localization of Pas7p and, in addition, one would expect a somewhat less efficient growth on oleate due to cytosolic depletion. This is exactly what Zhang and Lazarow reported. In fact, our results of subperoxisomal fractionation of that part of Pas7p which is associated with peroxisomes suggested that peroxisomal Pas7p is partly membrane bound (peripheral and integral) and behaves partly as if it were soluble in the matrix. In addition, the PTS2-specific binding of thiolase by Pas7p argues against a chaperone-like function of the latter, since neither mitochondrial Hsp70 nor BiP specifically bind targeting sequences.

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Common recombinant DNA techniques, including enzymatic modifications 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). All yeast transformations (except for introduction of the two-hybrid vectors into strain H7fc) were done by the method of Gietz and Sugino (1988). Total protein was measured by the BCA method (Fierce) using bovine serum albumin (BSA) as standard.

Cloning procedures

The oligonucleotides used are listed in Table II.

A 801 bp Smal–HindIII fragment coding for bacterial β-lactamase lacking its N-terminal targeting signal (Fabre et al., 1995) was ligated to similarly digested pEMBLexas (Mauri et al., 1993), creating pREP6/70. Both the FOX3 promoter and the 5′ 54 bp of the FOX3 open reading frame encoding the PTS2 were amplified by PCR using the oligonucleotides P1 and P2 and pREI56 (Erdmann, 1994) as template. The PCR product was digested with BamHI–EcoRI and cloned into pBLUESCRIPT SK+ (Stratagene) cut with the same enzymes. From this construct, a SalI–Smal fragment containing the FOX3 promoter and the 5′ 54 bp was transferred to similarly digested pRK670, creating pEMBL-PTS2β-lac (pFN-3/12).

A 1.9 kb BamHI–HindIII fragment was excised from pFN-3/12 and cloned together with a 0.3 kb HindIII–KpnI fragment comprising the CYC1 termination sequence (Zaret et al., 1982) into the BamHI–KpnI-digested CEN vector YCP1ac33 (Gietz and Sugino, 1988), resulting in YCP1PTS2β-lac (pFN-3/14).

Construction of the PTS2 mutant FOX3 allele (arginine at position 4 replaced by glycine) was carried out using the Altered Sites™ in vitro
mutagenesis system (Promega). For this purpose, the FOX3 gene was isolated from pRE156 (Erdmann, 1994), subcloned into pSELECT-I and mutated using primer NIS2 according to the manufacturer’s protocol. The mutagenized allele subsequently was introduced into YeP352 (Hill et al., 1986) that contained the cycl fusion termination sequence (pFN3/8).

From plasmid pWS-2 was constructed by introducing the FOX3 open reading frame and 227 bp promoter from pRE156 (Erdmann, 1994) into YeP352 with the cycl fusion termination sequence 3' of the gene. For expression of Pas7p from a plasmid with the LEU2 marker gene, an XbaI-Smal fragment was excised from pRSPT7/12 (Marzioch et al., 1994), comprising the Pas7 open reading frame, a 53 bp upstream and a 81 bp downstream region of the gene, and subsequently cloned into YeP351 (Hill et al., 1986) resulting in pPR7/4.

The first 18 amino acids comprising the PT2 of Fox3p were fused to the C-terminus of GST by amplifying the PT2-encoding 54 bp of FOX3 using primers Ku3 and P2 and pRE156 as a template. After cloning of the PCR fragment into pUC18 using the primer-derived Smal and EcoRI sites, the fragment was excised with BamHI and EcoRI and subsequently introduced into pGEX-4T3 (Pharmacia).

Antibodies

Anti-myc monoclonal antibodies (9E10) were a kind gift of M. Eilers (Heidelberg). Anti-thiolase (Fox3p) and anti-Pas3p antibodies were as reported (Erdmann and Kunau, 1994 and Höhfeld et al., 1991, respectively). Polyclonal antibodies against β-lactamase were kindly provided by R. Roggenkamp (Düsseldorf, Germany).

The Pas7 gene was amplified by PCR using plasmid SK’ P7/12 and primer M1 (Marzioch et al., 1994) and universal primer oligonucleotide, respectively. The PCR product was digested with BglII and EcoRI, cloned into pGEX-4T1 (Pharmacia) and digested with BamHI and EcoRI, creating pH57G. This fuses the N-terminal 195 amino acid residues of Pas7p to GST, under control of the tac promoter. The fusion protein was expressed in and purified from E.coli T1G1, according to the manufacturer’s instructions (Pharmacia).

Rabbit polyclonal antibodies to GST–Pas7p (1–195) were produced by Eurogentec (Seraing, Belgium) according to standard methods (Harlow and Lane, 1988). Antiserum were tested initially for their ability to react in Western blots to the GST–Pas7 (1–195) fusion protein that was present in whole cell extracts of E.coli T1G1 transformed with pH57G. Antibodies were affinity purified by binding to nitrocellulose derivatized with GST–Pas7p (1–375) fusion protein. The gene encoding this fusion protein was constructed by digestion of pH57G with BamHI and Smal and insertion of a BamHI-Smal PAS7 fragment excised from plasmid SK’ P7/12, resulting in plasmid pPR7/7 encoding a fusion of GST with the full-length Pas7p.

Nitrocellulose pieces with immobilized GST–Pas7p (1–375) fusion protein were incubated for 30 min at 30°C with antiserum against the GST–Pas7p (1–195) fusion protein. The nitrocellulose pieces were washed five times with TBS–Tweed [50 mM Tris–HCl (pH 8.0), 200 mM NaCl, 0.05% Tween 20] and bound protein was eluted with 0.1 M glycine–HCl (pH 2.5) and 1% BSA. Samples were neutralized with 1/4 volume of 1 M Tris–HCl (pH 7.5), dialysed against TBS–Tweed and assayed for their ability to recognize Pas7p on immunoblots of yeast lysates that were prepared by the method of Yaffe and Schatz (1984).

Western blotting

Proteins were separated by electrophoresis and transferred to nitrocellulose according to Harlow and Lane (1988). Protein–antibody complexes were visualized either by treatment with alkaline phosphatase colour developing reagents (Blake et al., 1984) or enhanced chemiluminescence (ECL, Amersham).

Electron microscopy and immunocytochemistry

Methods for the preparation of cells for electron microscopy have been described by Erdmann et al. (1989). Immunocytochemical experiments were performed on ultrathin sections of Lowicryl–embedded cells, using specific antibodies against β-lactamase as described by Douma et al. (1985).

Subcellular fractionation and enzyme assays

Yeast lysates were prepared and fractionated by differential centrifugation as described earlier (Erdmann et al., 1989). Further subfractionation by isopycnic sucrose density gradient centrifugation was performed by loading cell lysates onto a continuous 20–53% sucrose density gradient. Centrifugation, fractionation of the gradient and preparation of samples were carried out according to Höhfeld et al. (1991).

Hypotonic lysis of organelle preparations with 10 mM Tris–HCl pH 8.0 and carbonate extraction of peripheral membrane proteins with 0.1 M Na2CO3, pH 11.5 were performed according to Crane et al. (1994).

β-Lactamase was assayed as described (Cartwright et al., 1994) using PADAC (CalBiochem) as substrate. Acetyl-CoA acyltransferase (3-oxoacyl-CoA thiolase; EC 2.3.1.16), catalase (EC 1.11.1.6) and fumarate hydratase (fumarase; EC 4.2.1.2) were assayed as described by Moreno de la Garza et al. (1985) and Veenhuis et al. (1987).

For quantitative studies of β-galactosidase activity, yeast strains were grown on 0.3 % SD medium to stationary phase. Equal amounts of cells (500 mg) were resuspended in 500 µl of H buffer [10 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 0.5 mM diithiothreitol (DTT), 0.5 mM benzamidine and 1.2 mM phenylmethylsulphonyl fluoride (PMSF)]. The cells were disrupted by vortexing for 6 min (12×30 s) using 0.5 mm glass beads. Homogenates were clarified by centrifugation at 26 000 g. The supernatant was used subsequently for the assay (Rose et al., 1990).

Co-immunoprecipitation

Co-immunoprecipitation

Yeast cells were grown on 0.3 % SD medium to late log phase and subsequently for 15 h in YNGO (0.1% glucose, 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract and 0.67% yeast nitrogen base). Induction of the CUP1 promoter was performed with 0.025 g/l CuSO4 according to Marzioch et al. (1994). The cells were washed with dH2O and stored at −70°C. For each immunoprecipitation experiment, 0.8–1.0 g of cells were used. Three ml of solution A (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.2% Triton X-100), protease inhibitors [0.02% PMSF (Serva), 15 µg/ml benstatin, 1.5 µg/ml pepstatin, 1 µg/ml leupeptin, 0.1 µg/ml chymostatin (Boehringer Mannheim)] and 3 g of glass beads (0.5 mm) were added to the cells. Breakage was achieved by vortexing for 4 min (8×30 s with breaks of at least 30s on ice) (Lamb et al., 1994). Samples were filtered through cotton wool and liquid was transferred to Corex tubes and centrifuged at 35 000 g for 30 min. Extracts normalized for protein and volume were added to 5 µl of α-myc IgG (9E10) bound to sheep α-mouse IgG Dynabeads (Dynal) and incubated for 2 h at 4°C. Samples were washed three times with 1 ml of solution A and subsequently resuspended in 60 µl of Laemmli sample buffer.

Preparation of anti-myc antibodies bound to anti-mouse IgG Dynabeads was accomplished as follows: per immunoprecipitation, 50 µl of anti-mouse IgG Dynabeads were blocked with 5% BSA in phosphate-

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**Table II. List of oligonucleotides used in this study**

<table>
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<tr>
<th>Name</th>
<th>Oligonucleotide sequence</th>
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<tr>
<td>P1</td>
<td>5'-TATATGGATCCGTCACGATAGG-3'</td>
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<tr>
<td>P2</td>
<td>5'-TGAATTCCCCGGACCATGCGCTTCAC-3'</td>
</tr>
<tr>
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<td>5'-CCATCTGACCACGCTACGATCTACATATG-3'</td>
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</tr>
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</tr>
</tbody>
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buffered saline (PBS) buffer for 2 h, washed five times with 10 vol of PBS and saturated with anti-myc ascites fluid (9E10) at 4°C overnight. The supernatant was removed, beads were washed five times with 1 ml of solution A, resuspended in 50 μl of solution A and subsequently used for the precipitation experiment.

Two-hybrid plasmids and assays

The PAS7 open reading frame (+1 to +1128) was amplified from pRS7/1.2 (Marzioch et al., 1994) with the upstream primer KU1 and the downstream primer KU2. The resulting PCR fragment was cloned into the Clal and BamHI site of pBluescript SK+ (Stratagene) by using the primer-derived Clal and BclI sites, resulting in pEW1. To avoid errors due to Taq polymerase, the 586 bp PAS7 internal EcoRV–HindIII fragment was replaced by the wild-type sequence and flanking regions were re-synthesised. The PCR fragment was isolated by using the vector, SacI and Sall site and subsequently cloned into the GAL4 DNA binding domain vector pCP86 (Chevray and Nathans, 1992) and GAL4 activation domain vector pPC97 (Chevray).

The entire FOX3 open reading frame was amplified by PCR from pRE136 (Erdmann, 1994) using KU3 as sense primer and KU5 as antisense primer. To give rise to a FOX3 truncated of the PTS2 primers, KU4 and KU5 were used. The PCR products were cloned into pBluescript SK+ using the primer-derived SalI and BclI sites. Further cloning of both inserts was performed by SalI–SacI digestion and introduction of the fragments into pCP86 and pCP97.

To generate GAL4 DNA binding domain fusions and GAL4 activating domain fusions of β-lactamase and P2TS2–β-lactamase in vectors pCP97 and pCP86, β-lactamase was amplified via PCR using E1 and E2 primer from pRS6/68 and P2TS2–β-lactamase by using KU4 and E2 primers and pFN-3/12 as template. PCR products were cloned into pBluescript SK+ via the SalI and SacI sites that had been introduced into the primers. The same fragments were taken from the pBluescript SK+ constructs and subsequently ligated into the pPC vectors. pRF6/68 contained a mutated β-lactamase (Fabre et al., 1995) gene with an XbaI site following the C-terminal coding region in pBluescript II SK+.

In order to clone the human peroxisomal 3-oxoacyl-CoA thiolase into pCP86, the open reading frame was amplified from a human cDNA library (Clonetech) by using the primers KU47 and KU48. After subcloning of the PCR product into pEW1 using the SacI and Sall sites that had been introduced into the primers and thus replacing the PAS7 insert, further cloning was performed by isolating the thiolase cDNA insert from pBluescript SK+ via SalI–SacI digestion and ligation of the fragment into pCP86.

The PAS10 fused to the GAL4 activation domain in pCP86 (Elsgersma et al., 1995) was kindly provided by H.Tabak.

For transforming the yeast strain HF7c (Clonetech), we followed the Matchmaker protocol supplied by the manufacturers. Double transformants were selected on SD medium lacking tryptophan and leucine. Colonies were transferred to a nitrocellulose filter which was immersed in liquid nitrogen for 10–20 s. The filters were allowed to come to room temperature, then placed on top of Whatmann 3MM paper that had been soaked with 100 mM KP4 pH 7.0 containing 1 mg/ml X-Gal. Filters were incubated for 30 min to overnight at 30°C.

To assay for the expression of HIS3 as an additional reporter in strain HF7c, cells were transferred to SD plates lacking tryptophan, leucine and histidine but containing 10 mM 3-aminotriazol.

In vitro binding assay

For the in vitro binding assay, GST and GST–P2TS2 were isolated from E.coli T71 by affinity purification on glutathione–Sepharose 4B according to the manufacturer’s instructions.

The mycPas7p was expressed in and isolated from fos3-24A cells by immunoprecipitation (see above) using Dynabeads and anti-myc antibodies. After extensive washing with solution A, the beads were incubated with either GST or GST–P2TS2 for 2 h at 4°C. Beads were reprecipitated, washed with solution A and subsequently subjected to denaturing SDS–PAGE and Western blotting.

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References


S.cerevisiae PTS2 receptor gene


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