Tetratricopeptide repeat domain of *Yarrowia lipolytica* Pex5p is essential for recognition of the type 1 peroxisomal targeting signal but does not confer full biological activity on Pex5p

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Peroxins are proteins required for peroxisome assembly and are encoded by the *PEX* genes. The *Yarrowia lipolytica pex5-1* mutant fails to import a subset of peroxisomal matrix proteins, including those with a type 1 peroxisomal targeting signal (PTS1). Pex5p family members interact with a PTS1 through their characteristic tetratricopeptide repeat (TPR) domain. We used binding assays *in vitro* to investigate the nature of the association of *Y. lipolytica* Pex5p (*Yl*Pex5p) with the PTS1 signal. A purified recombinant *Yl*Pex5p fusion protein interacted specifically, directly and autonomously with a protein terminating in a PTS1. Wild-type *Yl*Pex5p translated *in vitro* recognized functional PTS1s specifically. This activity is abrogated by the substitution of an aspartic residue for a conserved glycine residue in the TPR domain (G455D) of *Yl*Pex5p encoded by the *pex5-1* allele. Deletion analysis demonstrated that an intact TPR domain of *Yl*Pex5p is necessary but not sufficient for both interaction with a PTS1 and functional complementation of a strain lacking *Yl*Pex5p.

Key words: organelle biogenesis, peroxin, peroxisome.

INTRODUCTION

Peroxisomes are ubiquitous organelles. Peroxisomal proteins are encoded by nuclear genes and are synthesized on cytosolic polysomes before being imported into peroxisomes post-translationally [1]. So far, three peroxisomal targeting signals (PTSs) have been identified (reviewed in [2,3]). PTS1, the most common signal, is a conserved C-terminal tripeptide with the sequence SKL (one-letter amino acid codes) or conserved variants thereof. PTS2, a much less common signal, is a degenerate nonapeptide found at or near the N-terminus of a few proteins and is sometimes part of a cleaved presequence. The mPTS is a less well characterized hydrophilic motif found on some peroxisomal integral membrane proteins. Some peroxisomal proteins do not contain any of the PTSs so far identified and the mechanism(s) of their targeting are unknown.

Factors that recognize PTS1 or PTS2 have been identified in several species. PTS1 receptors are encoded by the *PEX5* gene family [4–17]. These peroxins (proteins involved in peroxisome assembly) are characterized by a tetratricopeptide repeat (TPR) domain in the C-terminal half of the protein. TPR domains consist of tandem arrays of a degenerate 34-residue motif. These domains are found in proteins with many diverse roles and have been implicated in protein–protein interactions [18]. Pex5 proteins have been localized to different subcellular compartments, i.e. primarily cytosolic [5,10,11,17,19], associated with the outer side of the peroxisomal membrane [4,9,20], in the peroxisomal matrix [12] and in both the cytosol and peroxisomes [8]. The *PEX7* gene family encodes the PTS2 receptor [21–27]. Whether Pex7p is primarily cytosolic [21,23,26,28] or primarily peroxisomal [22,29] is also unclear. A shuttling model of PTS receptor function has been proposed to explain the different localizations reported for these peroxins [30]. Although Pex5p- and Pex7p-dependent targeting are independent processes in lower eukaryotes, both PTS receptors associate with a common network of docking factors including Pex13p, Pex14p and Pex17p at the peroxisomal membrane [19,31–37]. Mammalian Pex5p and Pex7p have recently been reported to interact with Pex14p [38-41].

Loss of function of either PTS receptor leads to disease states in humans, causing neonatal adrenoleucodystrophy and Zellweger syndrome in Pex5p mutation [11] and rhizomelic chondrodysplasia punctata in Pex7p deficiency [23–25]. Interestingly, import of proteins by the PTS1 and PTS2 pathways is not entirely independent in mammalian cells. The human [9–11,42], mouse [42] and Chinese hamster [15] *PEX5* genes each encode two forms of the protein that differ by the presence or absence of a 37-residue insertion N-terminal to the TPR domain. The short form of Pex5p can functionally complement the PTS1 import defect in *pex5* mutant cells but only the long form of the protein can also rescue the PTS2 import defect that is an additional feature of some *pex5* cells [15,42].

The *Yarrowia lipolytica pex5-1* (formerly *pay32-1*) mutant strain was identified as part of a genetic screen for mutants of peroxisome biogenesis [12]. *Y. lipolytica* strains in which Pex5p is mutated, or from which it is absent, have morphologically abnormal peroxisomes and fail to import a subset of peroxisomal matrix proteins, including those that are immunoreactive with anti-SKL antiserum. Most soluble proteins destined for peroxisomes are mislocalized to the cytosol or are found trapped in the peroxisomal membrane at an intermediate stage of import. For example, a 62 kDa anti-SKL-reactive protein was found stuck in the membrane with its immunoreactive portion in the peroxisomal matrix. In contrast, the import of thiolase (a PTS2-containing protein) into the peroxisomal matrix is normal in *pex5* mutants and none of this protein is trapped at the membrane. The *Y. lipolytica PEX5* gene encodes a 598-residue protein, *Yl*Pex5p, with high amino acid sequence similarity to PTS1 receptors from other species, especially in the TPR domain. In wild-type cells, *Yl*Pex5p is localized primarily to the inner side

Abbreviations used: GST, glutathione S-transferase; MBP, maltose-binding protein; ORF, open reading frame; PTS, peroxisomal targeting signal; TPR, tetratricopeptide repeat.

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of the peroxisomal membrane but approx. 30 % of the protein is found in the peroxisomal matrix. Of the matrix pool of *Yl*Pex5p, most is complexed with anti-SKL-reactive proteins. The *pex5-1* mutant produces an apparently full-length *Yl*Pex5p that is imported into peroxisomes but does not associate with the membrane and therefore does not complex with anti-SKL-reactive proteins. *Yl*Pex5p has been proposed to be an intra-organellar component of the peroxisomal matrix translocation machinery [12].

In the present study we present evidence that *Yl*Pex5p can specifically, directly and autonomously recognize PTS1 sequences. We report on the nature of the *pex5-1* mutation and its effect on PTS1 binding. A detailed deletion analysis reveals that the TPR domain of *Yl*Pex5p is necessary but not sufficient for PTS1 binding or for functional complementation of a strain lacking *Yl*Pex5p.

MATERIALS AND METHODS

Strains and media

The yeast strains used in this study are listed in Table 1. Yeast cells were grown at 30 °C in complete (YEPD) or minimal (YNA, YNO, 2 × CMD, 2 × CMO) medium, as required. YEPD and YNO have been described [12]. YNA contained 0.67 % yeast nitrogen base without amino acids (Difco, Detroit, MI, U.S.A.) plus 2 % (w/v) sodium acetate. YNA and YNO were supplemented with leucine, uracil and lysine, each at 50 μg/ml, as required. 2 × CMD contained 1.34 % (w/v) yeast nitrogen base without amino acids, complete supplement mixture (minus leucine and uracil, as appropriate) at twice the concentration recommended by the manufacturer (BIO 101, Vista, CA, U.S.A.) plus 2 % (w/v) glucose. 2 × CMO contained 1.34 % yeast nitrogen base without amino acids, complete supplement mixture (minus leucine and uracil, as appropriate) at twice the recommended concentration, plus 0.05 % (v/v) Tween 40 and 0.1 % (v/v) oleic acid.

Sequencing the *pex5-1* allele and creation of the *pex5-G455D* strain

Genomic DNA isolated from *Y. lipolytica* wild-type and *pex5-1* strains was used as a template for PCR with primers 32RI5′ and 32RI3′ (Table 2). PCR products were made blunt with the Klenow fragment of DNA polymerase I (Klenow) and cloned into the *Sma*I site of pGEM7Zf(+) (Promega, Madison, WI, U.S.A.).

Site-directed mutagenesis of the *PEX5* gene was used to direct a Gly → Asp substitution at codon 455. A *Hin*dIII fragment of *Y. lipolytica* genomic DNA including the entire *PEX5* gene [12] was

Table 1 Yeast strains used in this study

Strain *E122* was provided by C. Gaillardin (Thiverval-Grignon, France); strains *E:GDU3* and *pex5-G455D* were from the present study.

Strain	*Genotype*	Reference
E122	*MATA, ura3-302, leu2-270, lys8-11*	
pex5-1	*MATA, ura3-302, leu2-270, lys8-11, pex5-1*	[12]
pex5-KO	*MATA, ura3-302, leu2-270, lys8-11, pex5::LEU2*	[12]
E:GDU3	*MATA, ura3-302, leu2-270, lys8-11, PEX5::pGDU3(pex5-G455D, URA3)*	
pex5-G455D	*MATA, ura3-302, leu2-270, lys8-11, pex5-G455D*	

Table 2 Oligonucleotides used in this study

Relevant restriction endonuclease sites or compatible overhangs are underlined in the sequences (see the Materials and methods section).

Name	Sequence
32RI5′	5′-ATTGAATTCCAGCGCGGTAGTTGC-3′
32RI3′	5′-ATTGAATTCTAATAACCTTGGGCCTCC-3′
G455D	5′-CGTTTCCGTAGAACAGAACATCTAGACCAACTTGGACATCAGCG-3′
EQ1	5′-GATCCCCGC-3′
EQ2	5′-AATTGCGGG-3′
SKL1	5′-TCGATCCAAGCTTTGA-3′
SKL2	5′-GGCCTCAAAGCTTGGA-3′
LKS1	5′-TCGACTTAAGTCCTGA-3′
LKS2	5′-GGCCTCAGGACTTAAG-3′
AKI1	5′-TCGAGCAAAGATCTGA-3′
AKI2	5′-GGCCTCAGATCTTTGC-3′
SKLS1	5′-TCGATCCAAGCTTTCCTGA-3′
SKLS2	5′-GGCCTCAGGAAAGCTTGGA-3′
KK	5′-CCTCTCATAAACGACATGGTGGTACCTCCTGCTTGCTTTGG-3′
KKBT	5′-ATTGGTACCACTATGGGTATGGGGGGAATGCCCATG-3′
KKCT	5′-ATTGGTACCACCATGGAGCACCCTGATCCCTTC-3′
KKET	5′-ATTGGTACCACCATGGGCAACGAGGATAAGTTCCAG-3′
KKREV	5′-GGCCAATAATCCATGGCTGC-3′
BIAT	5′-ATTTACGTACTAGTGCTCCATGAACTGGTTG-3′
BIDT	5′-ATTTACGTACTATCGGGCCTGGTCCACAACC-3′
BIFT	5′-ATTTACGTACTACTCAACCTTGTGCATGGAC-3′

ligated into plasmid pRS425 [43] to yield plasmid p425PEX5, in which the orientation of the *PEX5* open reading frame (ORF) was the same as that of the ORF of the *LacZ* gene. p425PEX5 was transformed into *Escherichia coli* TG1 cells, from which single-stranded DNA was isolated. Single-stranded DNA was used as the template for site-directed mutagenesis with the mutant oligonucleotide G455D (Table 2). Mutagenesis products were transformed by electroporation into *E. coli* and analysed by digestion with *Xba*I and DNA sequencing. Plasmid p32ΔX is a pBluescriptSKII(−) (Stratagene, La Jolla, CA, U.S.A.) vector construct with its *Xho*I site destroyed and containing the wild-type *PEX5* gene at its *Eco*RI site. A 352 bp *Nde*I–*Xho*I fragment of p425G455D containing the engineered mutation was used to replace the equivalent wild-type sequence of p32ΔX to generate plasmid pGDΔX. A *Sal*I fragment containing the *Y. lipolytica URA3* gene was made blunt and ligated into a blunt-ended *Cla*I site of pGDΔX to yield the integrative plasmid pGDU3.

pGDU3 was used for pop-in/pop-out gene replacement [44] at the *PEX5* locus. *Y. lipolytica E122* cells were transformed by electroporation with *Bgl*II-digested pGDU3 and plated on YNA. Ura⁺ colonies were screened to test for site-specific integration of the plasmid and maintenance of the desired mutation. Strain *E:GDU3*, which contains one wild-type and one mutant copy of the *PEX5* gene flanking the *URA3* gene (Table 1), was grown in YEPD to relax uracil selection. Cells were plated on YNA containing 0.075 % 5-fluoro-orotic acid to select against cells with a functional *URA3* gene. Colonies resistant to 5-fluoro-orotic acid were screened to find a strain, *pex5-G455D* (Table 1), that had lost the *URA3* marker by homologous recombination between the *PEX5* genes and had retained the mutant allele.

Generation of plasmids encoding *Yl*Pex5p and variants

The pKK series of plasmids was prepared in pBluescriptSKII(−) for the synthesis of *Yl*Pex5p and variants *in vitro*. Single-stranded DNA from p425PEX5 was used as a template for site-directed mutagenesis with oligonucleotide KK (Table 2) to create a

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consensus site for mammalian translational initiation [45] and a *Kpn*I site upstream of the initiation codon of the *PEX5* gene. The resulting plasmid, p425KKII, was digested with *Kpn*I and *Cla*I to yield an insert that was ligated into pBluescriptSKII(−) to make plasmid pBSKK. A *Sph*I fragment from p425PEX5 or pGDΔX (consisting of nt 139–1873 of the wild-type *PEX5* or mutant *pex5-G455D* genes respectively) was exchanged against the equivalent region of pBSKK to generate plasmids pKKWT and pKKGD respectively.

Genes encoding truncated variants of *Yl*Pex5p were created by PCR, with the genomic clone of *PEX5* as template. To create N-terminal truncations, oligonucleotide KKBT, KKCT or KKET (Table 2) was used as the upstream primer; oligonucleotide KKREV (Table 2) was used as the downstream primer. PCR products were ligated into the *Eco*RV site of pGEM5Zf(+) (Promega) to make plasmids pG5BT, pG5CT and pG5ET respectively. A 268 bp *Kpn*I–*Eco*RV fragment of pG5BT, a 57 bp *Kpn*I–*Sal*I fragment of pG5CT and a 281 bp *Kpn*I–*Xho*I fragment of pG5ET were exchanged against the equivalent regions of pKKWT to generate plasmids pKKBT, pKKCT and pKKET respectively. To make C-terminal truncations of *Yl*Pex5p, oligonucleotide 32RI5′ was used as the upstream primer and oligonucleotide BIAT, BIDT or BIFT (Table 2) was used as the downstream primer, thereby creating a termination codon in the *PEX5* gene followed by a *Sna*BI site. PCR products were digested with *Apa*I and *Sna*BI and ligated into pRcCMV (Invitrogen, Carlsbad, CA, U.S.A.) to give plasmids pRcA, pRcD and pRcF respectively. A 189 bp *Eco*RV–*Sna*BI fragment of pRcA was ligated into pKKWT digested completely with *Stu*I and partly with *Eco*RV to give plasmid pKKAT. A 66 bp *Nde*I–*Sna*BI fragment of pRcD was ligated into pKKWT digested with *Nde*I and *Stu*I to give plasmid pKKDT. A 128 bp *Xho*I–*Sna*BI fragment of pRcF was ligated into pKKWT digested with *Xho*I–*Stu*I to give plasmid pKKFT. Plasmid pKKBF was created by exchanging the *Kpn*I–*Xho*I fragment of pKKFT for the equivalent region of pKKBT. Plasmid pKKCF was created by exchanging the *Kpn*I–*Nde*I fragment of pKKFT with the equivalent region of pKKCT.

The *PEX5* promoter was used to drive the expression *in vivo* of genes encoding truncated *Yl*Pex5p variants. The *Hin*dIII fragment of p425KKII was ligated into pBluescriptSKII(−) such that the direction of transcription of the *PEX5* gene was opposite that of the *LacZ* gene. This created plasmid pBSHK containing approx. 1 kbp of *Y. lipolytica* genomic DNA 5′ to the initiation codon of *PEX5* located between *Kpn*I sites. The *Kpn*I fragment of pBSHK was ligated into the *Kpn*I sites (in the correct orientation with respect to the ORFs) of the appropriate pKK plasmid to give plasmids pKPAT, pKPDT, pKPFT, pKPBT, pKPCT, pKPET and pKPBF respectively. The promoter–ORF combinations were liberated from the pKP plasmids with *Hin*dIII and ligated into the *E. coli*/*Y. lipolytica* shuttle vector pINA443 (*ARS68*, *URA3*; a gift from C. Gaillardin, Thiverval-Grignon, France) to give plasmids p443AT, p443DT, p443FT, p443BT, p443CT, p443ET and p443BF respectively. Expression of the wild-type *PEX5* gene was from plasmid p443KK, consisting of the *Hin*dIII fragment of plasmid p425KKII in pINA443. Plasmids were introduced into the *pex5-KO* strain by electroporation.

PTS1 binding assays *in vitro*

Variants of glutathione S-transferase (GST) modified at their C-termini were encoded by a series of plasmids based on pGEXQ, a derivative of pGEX4T1 (Amersham–Pharmacia Biotech). Complementary oligonucleotides EQ1 and EQ2 (Table 2) were ligated into pGEX4T1 digested with *Bam*HI and *Eco*RI to yield plasmid pGEXQ, in which the *Eco*RI site of pGEX4T1was destroyed, thereby creating a codon for glutamine (CAA) in place of that for glutamic acid (GAA). Plasmids pGEXQ-SKL, pGEXQ-LKS, pGEXQ-AKI and pGEXQ-SKLS (encoding GST–SKL, GST–LKS, GST–AKI and GST–SKLS respectively) were made by ligating annealed oligonucleotide pairs SKL1 and SKL2, LKS1 and LKS2, AKI1 and AKI2, and SKLS1 and SKLS2 respectively (Table 2) into pGEXQ digested with *Sal*I and *Not*I. The sequences of the C-termini of the encoded GST variants are LVPRGSPQFPGR(SKL/LKS/AKI/SKLS) respectively. The pGEXQ variants were transformed into protease-deficient *E. coli* BLR(DE3) cells (Novagen, Madison, WI, U.S.A.). Cells were disrupted by incubation with B-PER bacterial protein extraction reagent (Pierce, Rockford, IL, U.S.A.). Cell extracts were passed over glutathione–Sepharose to isolate GST proteins. Bound material was eluted with 10 mM reduced glutathione in 50 mM Tris/HCl, pH 8.0. Eluted proteins were dialysed at 4 °C for 4 h against P8BB binding buffer [20 mM HEPES/KOH (pH 7.3)/110 mM potassium acetate/5 mM sodium acetate/2 mM magnesium acetate/1 mM EDTA/2 mM dithiothreitol] [4] containing 20 % (v/v) glycerol, then stored at −80 °C until needed.

pMBP5 encodes MBP–PEX5, a recombinant protein consisting of all except the first 46 residues of *Yl*PEX5p fused to the C-terminus of *E. coli* maltose-binding protein (MBP) [12]. pMBP5 and pMALc-2 (New England Biolabs, Mississauga, Ontario, Canada) were used to produce MBP–PEX5 and MBP respectively in *E. coli* BLR(DE3) cells. Cells were disrupted, and MPB-PEX5 and MBP were purified on amylose resin, then dialysed and stored as described above.

Glutathione–Sepharose beads were mixed with an equal volume of P8BB buffer containing 20 % (v/v) glycerol and GST-variant protein chimaera at a concentration of 7.5 μg/μl at room temperature for 30 min to saturate GST-binding sites. After extensive washing with P8BB containing 10 % (v/v) glycerol, 20 μl of loaded beads was mixed with 200 μl of blocking buffer [P8BB containing 10 % (v/v) glycerol, 0.2 % (v/v) Nonidet P40 and BLR(DE3) cell extract at 10 μg/μl] to block non-specific protein-binding sites. MBP–PEX5 (5 μg) or an equimolar amount of MBP was mixed with 200 μl of blocking buffer, incubated on ice for 30 min and centrifuged at 16000 ***g*** for 10 min. The supernatant was added to the bead mixture and incubated at room temperature for 90 min with gentle mixing. Beads were centrifuged at 500 ***g*** for 5 min, after which the supernatant was removed. Beads were then washed with 400 μl of each of (1) P8BB containing 10 % (v/v) glycerol, 0.2 % (v/v) Nonidet P40 and 2 μg/μl BSA, (2) P8BB containing 10 % (v/v) glycerol, 0.1 % (v/v) Nonidet P40 and 2 μg/μl BSA, and (3) P8BB containing 10 % (v/v) glycerol. Wash buffers were ice-cold; centrifugations were performed at 4 °C. Washed beads were resuspended in 20 μl of SDS/PAGE sample buffer and boiled for 5 min. Eluted proteins were analysed by SDS/PAGE, followed by staining with Coomassie Blue. All blocking and wash buffers contained protease inhibitors [leupeptin, pepstatin and aprotinin (each at 1 μg/ml), 0.5 mM benzamidine hydrochloride, 5 mM NaF and 1 mM PMSF]. In some experiments (see the Results section), *E. coli* cell extract and BSA were omitted from the blocking buffer and wash buffers respectively.

Synthesis of *Yl*Pex5p variants *in vitro* was performed with a coupled reticulocyte lysate system for transcription/translation *in vitro* with T7 RNA polymerase. [^{35}S]Methionine incorporation into protein was assessed by precipitation with trichloroacetic acid. An aliquot of lysate corresponding to 50000 precipitated radioactive counts of wild-type *Yl*Pex5p, or an aliquot containing

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the number of radioactive counts from an equimolar amount of a *Yl*Pex5p variant (unless specified otherwise), was brought to a final volume of 2 μl (unless specified otherwise) with unprogrammed reticulocyte lysate that had been diluted 1 : 1 with water. The diluted mixture was processed for binding as described above for MBP proteins, except that blocking buffer contained 10 μg/μl BSA instead of *E. coli* lysate. Proteins released from beads by being boiled in SDS/PAGE sample buffer were analysed by SDS/PAGE and fluorography. Dried gels were exposed to preflashed Kodak XAR-5 X-ray film at −80 °C.

Analytical procedures

Yeast lysates were prepared by disruption of cells with glass beads. Protein concentration was determined with a commercially available kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada) with BSA as the standard. Immunoblotting was performed essentially as described [46]. Immunodetection was by enhanced chemiluminescence (Amersham–Pharmacia Biotech) with guinea-pig anti-*Yl*Pex5p primary antibodies [12] and secondary horseradish peroxidase-conjugated goat anti-(guinea-pig) IgG. Computer alignment of protein sequences was performed with the CLUSTAL program.

RESULTS

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

We tested the ability of *Yl*Pex5p to recognize a PTS1 tripeptide by using a PTS1 binding assay *in vitro* modelled on that of McCollum et al. [4]. GST variants ending in a PTS1 (GST–SKL) or the reverse tripeptide LKS (GST–LKS), which does not resemble a PTS1 but maintains its charge density, were immobilized on glutathione–Sepharose beads. MBP–PEX5, a chimaera consisting of *Yl*Pex5p lacking its first 46 residues fused to the C-terminus of MBP, was retained on GST–SKL beads but not on GST–LKS beads (Figure 1A). MBP alone was not retained on either GST–SKL or GST–LKS beads (Figure 1A). Therefore the MBP–PEX5 fusion protein had specific PTS1-binding activity located in its *Yl*Pex5p domain. The specificity of this interaction is further shown by the fact that MBP–PEX5 was enriched from a complex mixture of proteins, an *E. coli* lysate, by GST–SKL beads. Binding reactions were repeated with MBP–PEX5 and MBP in the absence of any additional protein during the incubation or washing steps (Figure 1B). MBP–PEX5 was still retained specifically on the GST–SKL beads, suggesting that the interaction between *Yl*Pex5p and PTS1 is direct and does not require other factors.

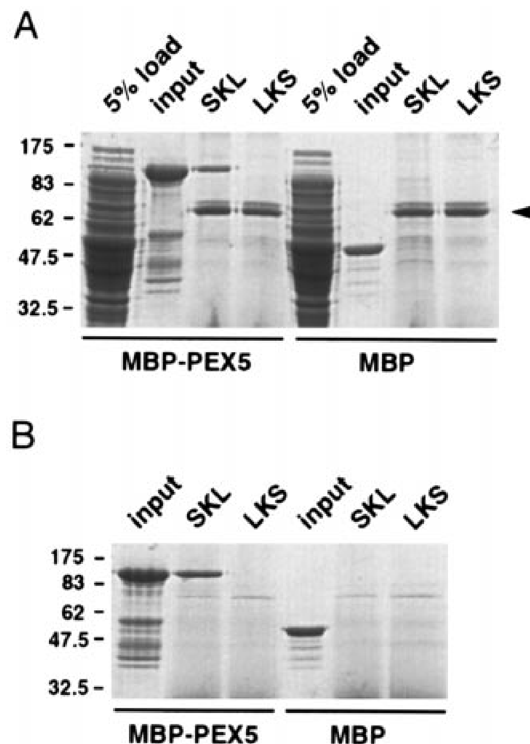

Figure 1 *Yl*Pex5p-MBP chimaera recognizes the PTS1 sequence

GST–SKL (lanes SKL) and GST–LKS (lanes LKS) purified from bacteria were immobilized on glutathione–Sepharose and incubated with bacterially produced, purified MBP–PEX5 protein chimaera or an equimolar amount of the control, unfused MBP. (**A**) MBP proteins and immobilized GST proteins were preblocked with buffer containing *E. coli* lysate before incubation. The first two wash buffers contained BSA (arrowhead). Bound proteins were eluted by boiling in SDS/PAGE sample buffer, separated by SDS/PAGE and revealed by staining with Coomassie Blue. An amount equivalent to 5% of the MBP–PEX5 or MBP preincubation reaction was loaded to show the starting protein profile (lanes 5% load). (**B**) MBP proteins and immobilized GST proteins were incubated together without preblocking. No additional proteins were added to the incubation or wash buffers. Bound proteins were eluted and analysed as described above. The total amount of MBP–PEX5 or unfused MBP used in the binding reaction is shown (input).

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

The *pex5-1* strain synthesizes an apparently full-length *Yl*Pex5p that is imported into peroxisomes but fails to associate with anti-SKL-reactive proteins [12]. The nature of the mutation in *pex5-1* cells was determined by PCR amplification and sequencing of the *PEX5* alleles from both *pex5-1* and wild-type strains. A single change from the wild-type *PEX5* gene was found in the *pex5-1* allele. A G → A transition at nt 1364 results in a Gly → Asp substitution at residue 455 of *Yl*Pex5p (Figure 2A). Gly-455 is located in the fifth TPR motif of *Yl*Pex5p; its functional importance is highlighted by the fact that glycine is conserved at this position in all known Pex5p family members (Figure 2B).

To demonstrate further that the *pex5-1* phenotype was due to the identified mutation, an allele of *PEX5* encoding Pex5p-G455D, with an aspartic residue at position 455, was created by site-directed mutagenesis. The mutant allele was used to replace the wild-type gene in the *E122* strain, generating the strain *pex5-G455D* (Table 1). The *pex5-G455D* strain manifests the same growth defect on oleate medium as the original mutant *pex5-1* and the *PEX5* gene disruption strain, *pex5-KO* [12] (Figure 3). These results confirm that the G455D substitution is sufficient to inactivate *Yl*Pex5p, thus preventing proper peroxisome assembly and function, including the utilization of fatty acids. Pex5p-G455D was synthesized at normal levels in the *pex5-G455D* strain (results not shown).

G455D mutation abrogates the PTS1-binding activity of *Yl*Pex5p

Full-length wild-type *Yl*Pex5p and Pex5p-G455D were tested for their ability to recognize variants of the PTS1 signal. The proteins were translated *in vitro* and labelled with [^{35}S]methionine to facilitate the detection of any weak interactions. Wild-type *Yl*Pex5p was specifically retained on GST–SKL and GST–AKI beads (Figure 4, WT). The C-terminal tripeptide AKI has been shown to function as a PTS1 in *Candida albicans* and *Saccharomyces cerevisiae* [47]; *Y. lipolytica* contains peroxisomal proteins that are immunoreactive with anti-AKI antiserum [48].

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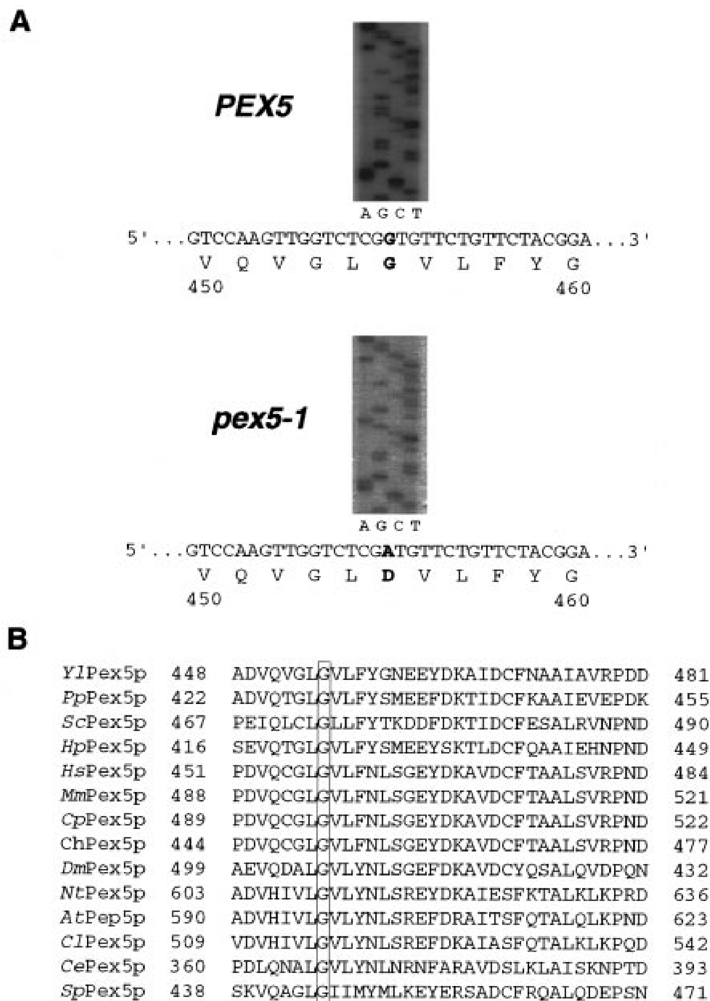

Figure 2 Sequence analysis of the *pex5-1* allele

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

*Yl*Pex5p was not retained either on GST–LKS beads, in which the C-terminal tripeptide does not conform to the PTS1 consensus, or on GST–SKLS beads, in which the canonical PTS1 is displaced from the extreme C-terminus by the addition of an extra amino acid, a construction that has been shown to prevent the targeting to peroxisomes of a protein normally resident in peroxisomes [49]. Therefore wild-type *Yl*Pex5p recognized only functional PTS1 tripeptides.

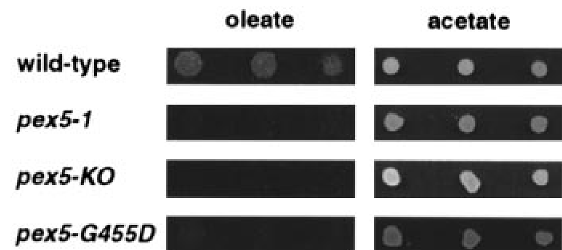

Figure 3 Growth of wild-type and *pex5* mutant strains on oleate and acetate

Strains *E122*, *pex5-1*, *pex5-KO* and *pex5-G455D* were pregrown in YEPD medium. Cultures were adjusted to the same attenuance; serial 2-fold dilutions were plated on YNO (containing oleate) or YNA (containing acetate). Growth was for 4 days (YNO) or 2 days (YNA) at 30 °C.

In contrast with the wild-type protein, Pex5p-G455D was not specifically retained on beads containing any of the PTS1 variants tested (Figure 4, G455D), suggesting that the Gly → Asp substitution prevented the mutant *Yl*Pex5p from binding to PTS1s. These results are in agreement with the previous finding that anti-SKL-reactive proteins associated with *Yl*Pex5p from wild-type cells but not with the mutant form of *Yl*Pex5p from *pex5-1* cells [12].

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

To delineate the region(s) of *Yl*Pex5p involved in PTS1 recognition, truncated variants of *Yl*Pex5p were labelled with [^{35}S]methionine by translation *in vitro* and assayed for their PTS1 binding activity. Deletions of regions N-terminal to the TPR domain did not significantly alter the specific PTS1 binding ability of truncated variants relative to that of wild-type *Yl*Pex5p (Figure 5; compare B and C with WT). These results support the observation that, in the context of a fusion chimaera, the first 46 residues of *Yl*Pex5p were not required for interaction with a PTS1 (Figure 1). In contrast, deletion of the N-terminus and the first half of the TPR domain of *Yl*Pex5p resulted in a variant with no PTS1 binding activity (Figure 5, E). C-terminally truncated variants that terminated either just before the TPR domain (Figure 5, A) or two residues into the fourth TPR motif (Figure 5, D) failed to show PTS1 binding activity. Removal of the region C-terminal to the TPR domain significantly decreased, but did not completely eliminate, PTS1 binding (Figure 5, F). This low level of binding was reproducible. Taken together, these results suggest that the N-terminal 300 residues of *Yl*Pex5p were completely dispensable for PTS1 binding, the C-terminal tail (residues 550–598) of *Yl*Pex5p was not required for PTS1 recognition but does increase the efficacy of the binding reaction, and the TPR domain of *Yl*Pex5p was essential for binding PTS1. Therefore the minimum PTS1-binding domain of *Yl*Pex5p is predicted to lie within residues 301–549. Surprisingly, a protein with these endpoints (Figure 5, CF) showed no PTS1 binding activity, even when present in a 4-fold molar excess over wild-type or mutant F proteins. However, a protein consisting of amino acids 155–549 of *Yl*Pex5p (Figure 5, BF) had a low level of PTS1 binding activity similar to that of mutant F, suggesting that the region between residues 155 and 300 also had a role in PTS1 recognition and/or in stabilization of the interaction between the targeting signal and its receptor.

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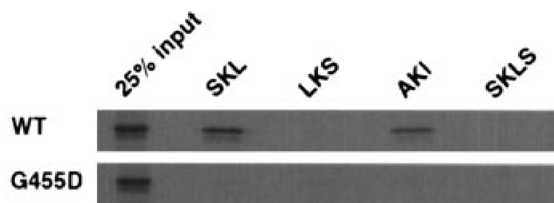

Figure 4 Analysis of wild-type *Yl*Pex5p and Pex5p-G455D PTS1 binding activities

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

The biological activity of *Yl*Pex5p variants was assayed by testing their capacity for functional complementation of the growth defect of the *pex5-KO* disruption strain on oleate medium (Figure 6). The *PEX5* promoter was used to drive the expression of the various variant *Yl*Pex5p gene constructs. Although all transformants displayed strong growth on medium containing acetate as the carbon source (Figure 6, bottom panels), which does not require functional peroxisomes for its metabolism, only the transformant carrying a plasmid encoding the wild-type *Yl*Pex5p showed robust growth on oleate medium (Figure 6, middle panels), which does require functional peroxisomes for its metabolism. The strain synthesizing mutant F, lacking only the C-terminal end of *Yl*Pex5p, grew slowly on oleate medium. No growth on oleate medium was observed for strains synthesizing any of the other *Yl*Pex5p variants or for the strain carrying the empty vector alone. Cell lysates of the various transformants were analysed by immunoblotting to determine whether they were producing the different *Yl*Pex5p variants (Figure 6, top panel). Appreciable quantities of *Yl*Pex5p variants were made in all cases, except for variant E, for which no signal was detected even after overexposure of the blot (results not shown). Variant E was probably unstable because a version of variant E with two copies of an influenza haemagglutinin epitope tag appended to its C-terminus could not be detected with the appropriate monoclonal antibody (results not shown). The reason for the absence of a signal for variant E was not investigated further. From the results of the growth assay (Figure 6) and the PTS1 binding data *in vitro* (Figure 5), it can be concluded that the ability to recognize a PTS1 is necessary but not sufficient to confer full biological activity on a *Yl*Pex5p variant.

DISCUSSION

Y. lipolytica and other yeast species have proved to be invaluable tools for the identification of components required for peroxisome assembly. The *PEX5* gene of *Y. lipolytica* was identified in a genetic screen for mutants of peroxisome biogenesis [12] and shows high amino acid sequence similarity to genes from other organisms that encode PTS1 receptors [4,6,9–11,14,20]. Although the original mutant strain *pex5-1* synthesizes a *Yl*Pex5p of normal size, unlike the wild-type *Yl*Pex5p, it is not found in association with anti-SKL-reactive proteins *in vivo* [12]. Analysis of the *pex5-1* allele revealed that the mutant strain produces a *Yl*Pex5p with an aspartic residue substituted for a conserved glycine residue at position 455 (Figure 2). The G455D substitution did not support PTS1 binding by *Yl*Pex5p in an *in vitro* assay (Figure 4); together with the lack of detectable PTS1 interaction *in vivo* [12], this suggests a cause for the mutant *pex5-1* phenotype. The results presented here are consistent with a model in which the *pex5-1* mutant fails to assemble peroxisomes correctly because of the inability of the mutant *Yl*Pex5p to recognize the PTS1 motif rather than because of its inability to interact with other components of the peroxisomal protein import machinery. Although the latter possibility could exist, given that most wild-type *Yl*Pex5p is associated with the peroxisomal membrane and *Yl*Pex5p from *pex5-1* cells is found exclusively in the peroxisomal matrix [12], any loss of interaction between the

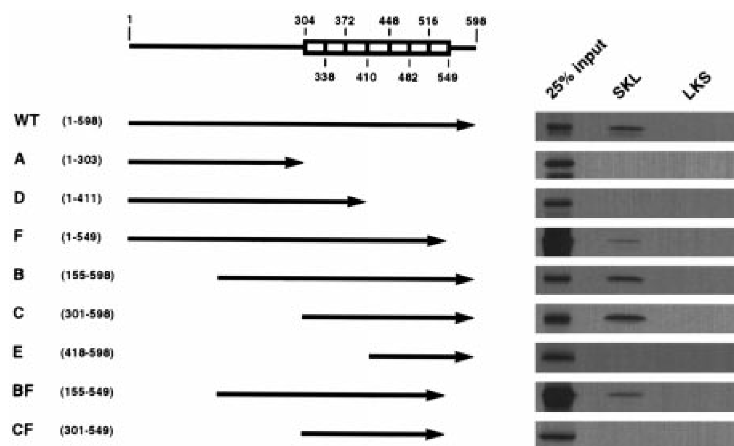

Figure 5 Domain analysis of PTS1 binding activity of *Yl*Pex5p

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

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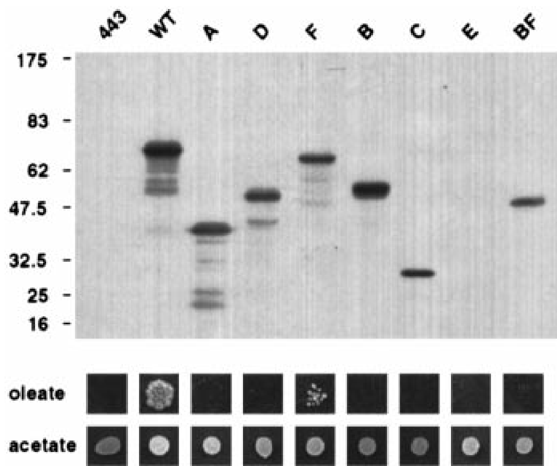

Figure 6 Growth characteristics of strains expressing wild-type and truncated forms of *Yl*Pex5p

Lanes are labelled to correspond with the constructs detailed in Figure 5. Top panel: *pex5-KO* cells carrying plasmid pINA443 (443) or pINA443 with an insert encoding wild-type (WT) or a particular truncated form of *Yl*Pex5p (A to BF) were precultured for 16 h in glucose-containing $2 \times$ CMD medium and then transferred to oleic acid-containing $2 \times$ CMO for 6 h. Cell lysates were prepared by disruption with glass beads. Each lysate (30 μg) was separated by SDS/PAGE and subjected to immunoblotting with anti-*Yl*Pex5p serum. The panel is a composite of two images: lanes B to BF were exposed for six times longer than lanes 443 to F. The numbers at the left indicate the positions of molecular size standards (in kDa). Bottom panels: *pex5-KO* strains carrying the plasmids described above precultured in $2 \times$ CMD, adjusted to the same cell density and plated on $2 \times$ CMO (oleate) or YNA (acetate). Growth was at 30 °C for 4 days on $2 \times$ CMO or 2 days on YNA.

mutant *Yl*Pex5p and other components of the peroxisomal translocation machinery seems to be a secondary effect of the inability of the mutant *Yl*Pex5p to recognize a PTS1 because a purified *Yl*Pex5p fusion protein is capable of binding to an SKL tripeptide without the participation of any other factor (Figure 1B).

TPR domains are predicted to form pairs of anti-parallel α-helices that interlock with the 'knobs' of one helix (formed by bulky residues such as tyrosine or phenylalanine) fitting into the 'holes' (formed by small amino acids such as glycine or alanine) of the adjacent helix [18,50]. The fact that the mutated amino acid residue in the *Yl*Pex5p of *pex5-1* cells is a conserved glycine implies that the structure of the TPR domain might be compromised in the mutant protein. Gly-455 is the eighth residue of the fifth TPR motif of *Yl*Pex5p. Not only is this residue conserved in Pex5p family members (Figure 2) but also, in general, a small amino acid is found in the eighth position of repeats of TPR-containing proteins with diverse functions [18]. Recently, two inactivating mutations in Chinese hamster Pex5p were found to involve substitutions of glutamic residues for glycine residues at position 298 in the first TPR motif and at position 485 in the sixth TPR motif [15].

Deletion analysis of *Yl*Pex5p was performed to define the regions needed for recognition of a PTS1. The N-terminal 300 residues of *Yl*Pex5p are not required for its association with a PTS1 because *Yl*Pex5p variants with deletions in this region showed approximately wild-type levels of PTS1 binding activity (Figure 5, WT, B and C). The TPR domain is essential for the interaction of *Yl*Pex5p with a PTS1 because every truncation tested in which part or all of the TPR domain was missing failed to show PTS1 binding activity (Figure 5, A, D and E). The C-terminal end (residues 550–598) of *Yl*Pex5p is not absolutely required for PTS1 recognition but its removal significantly decreases the PTS1 binding activity of *Yl*Pex5p (Figure 5, F and BF). Removal of the C-terminal end of *Yl*Pex5p also makes the region of residues 155–300 necessary for PTS1 binding because the TPR domain alone does not show PTS1 binding activity (Figure 5, CF). An intact TPR domain has been shown to be required for full PTS1 binding activity of *S. cerevisiae* Pex5p (*Sc*Pex5p) [6]. There seem to be residues critical for PTS1 binding outside the TPR motifs of *Sc*Pex5p because deletion of the C-terminal 48 residues abolishes PTS1 binding, and a *Sc*Pex5p variant that initiates 6 residues before the TPR domain has only very weak PTS1 binding activity. In contrast, the first three TPR motifs of *P. pastoris* Pex5p (*Pp*Pex5p) are sufficient and necessary for binding a PTS1 *in vitro* [20]. Truncation mutant D of *Yl*Pex5p (Figure 5) was designed to mimic the C-terminal deletion in the minimal PTS1-binding domain of *Pp*Pex5p (i.e. termination after the second amino acid of the fourth TPR motif) but no interaction was detected between mutant D and GST–SKL. It therefore seems that, in terms of the structural requirements for PTS1 binding, *Yl*Pex5p is more similar to *Sc*Pex5p than it is to *Pp*Pex5p.

The role of *Yl*Pex5p in peroxisome biogenesis is greater than simply its ability to bind a PTS1 tripeptide, because the extent of PTS1 binding by a *Yl*Pex5p variant (Figure 5) did not necessarily predict its ability to restore peroxisome function in a *pex5* gene disruption strain (Figure 6). Variants B and C (which lack the N-terminal 154 and 300 residues respectively of Pex5p) showed approximately wild-type levels of PTS1 binding activity but were unable to complement the *pex5-KO* strain for growth on oleic acid-containing medium. Mutant F, which lacks the C-terminal 48 residues of *Yl*Pex5p, showed weak PTS1 binding and was able to complement the *pex5-KO* strain partly. Mutant BF (residues 155–549) also bound GST–SKL weakly but did not complement the *pex5-KO* strain, reinforcing the functional importance of the N-terminal region of *Yl*Pex5p.

Why might a mutant *Yl*Pex5p that retains PTS1 binding ability not be fully biologically functional? Several reasons can be put forward. (1) The deleted region of *Yl*Pex5p could include information required to direct it to peroxisomes. *Yl*Pex5p does not have a recognizable PTS; the domain responsible for peroxisomal targeting has yet to be characterized. (2) The mutant *Yl*Pex5p might fail to interact with other components of the peroxisomal translocation machinery. Peroxin binding partners for *Yl*Pex5p await identification. (3) The mutant *Yl*Pex5p might not assume the correct quaternary protein structure. Purified human Pex5p produced in *E. coli* has recently been shown to exist as a homotetramer; the domain responsible for oligomerization was located within the N-terminal 213 residues [40]. (4) Truncated forms of *Yl*Pex5p might lack one or more domains needed for the release of PTS1-bearing proteins in a timely fashion, resulting in prolonged lives for complexes of *Yl*Pex5p and cargo proteins and preventing *Yl*Pex5p from recycling to pick up new cargo. Experiments are currently under way to address these issues.

In conclusion, we have demonstrated that *Yl*Pex5p can bind specifically, directly and autonomously with functional PTS1 motifs. We also have shown that an intact TPR domain of *Yl*Pex5p is necessary but not sufficient for both interaction with a PTS1 and functional complementation of a strain lacking *Yl*Pex5p.

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REFERENCES

1 Lazarow, P. B. and Fujiki, Y. (1985) Annu. Rev. Cell Biol. **1**, 489–530
2 Subramani, S. (1998) Physiol. Rev. **78**, 171–188
3 Hettema, E. H., Distel, B. and Tabak, H. F. (1999) Biochim. Biophys. Acta **1451**, 17–34
4 McCollum, D., Monosov, E. and Subramani, S. (1993) J. Cell Biol. **121**, 761–774
5 Van der Leij, I., Franse, M. M., Elgersma, Y., Distel, B. and Tabak, H. F. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 11782–11786
6 Brocard, C., Kragler, F., Simon, M. M., Schuster, T. and Hartig, A. (1994) Biochem. Biophys. Res. Commun. **204**, 1016–1022
7 Nuttley, W. M., Szilard, R. K., Smith, J. J., Veenhuis, M. and Rachubinski, R. A. (1995) Gene **160**, 33–39
8 Van der Klei, I. J., Hilbrands, R. E., Swaving, G. J., Waterham, H. R., Vrieling, E. G., Titorenko, V. I., Cregg, J. M., Harder, W. and Veenhuis, M. (1995) J. Biol. Chem. **270**, 17229–17236
9 Fransen, M., Brees, C., Baumgart, E., Vanhooren, J. C., Baes, M., Mannaerts, G. P. and Van Veldhoven, P. P. (1995) J. Biol. Chem. **270**, 7731–7736
10 Wiemer, E. A., Nuttley, W. M., Bertolaet, B. L., Li, X., Francke, U., Wheelock, M. J., Anne, U.K., Johnson, K. R. and Subramani, S. (1995) J. Cell Biol. **130**, 51–65
11 Dodt, G., Braverman, N., Wong, C., Moser, A., Moser, H. W., Watkins, P., Valle, D. and Gould, S. J. (1995) Nat. Genet. **9**, 115–125
12 Szilard, R. K., Titorenko, V. I., Veenhuis, M. and Rachubinski, R. A. (1995) J. Cell Biol. **131**, 1453–1469
13 Baes, M., Gressens, P., Baumgart, E., Carmeliet, P., Casteels, M., Fransen, M., Evrard, P., Fahimi, D., Declercq, P. E., Collen, D. et al. (1997) Nat. Genet. **17**, 49–57
14 Kragler, F., Lametschwandtner, G., Christmann, J., Hartig, A. and Harada, J. J. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 13336–13341
15 Otera, H., Okumoto, K., Tateishi, K., Ikoma, Y., Matsuda, E., Nishimura, M., Tsukamoto, T., Osumi, T., Ohashi, K., Higuchi, O. and Fujiki, Y. (1998) Mol. Cell. Biol. **18**, 388–399
16 Brickner, D. G., Brickner, J. H. and Olsen, L. J. (1998) Plant Physiol. **118**, 330
17 Wimmer, C., Schmid, M., Veenhuis, M. and Gietl, C. (1998) Plant J. **16**, 453–464
18 Goebl, M. and Yanagida, M. (1991) Trends Biochem. Sci. **16**, 173–177
19 Elgersma, Y., Kwast, L., Klein, A., Voorn-Brouwer, T., van den Berg, M., Metzig, B., America, T., Tabak, H. F. and Distel, B. (1996) J. Cell Biol. **135**, 97–109
20 Terlecky, S. R., Nuttley, W. M., McCollum, D., Sock, E. and Subramani, S. (1995) EMBO J. **14**, 3627–3634
21 Marzioch, M., Erdmann, R., Veenhuis, M. and Kunau, W.-H. (1994) EMBO J. **13**, 4908–4918
22 Zhang, J. W. and Lazarow, P. B. (1995) J. Cell Biol. **129**, 65–80
23 Braverman, N., Steel, G., Obie, C., Moser, A., Moser, H., Gould, S. J. and Valle, D. (1997) Nat. Genet. **15**, 369–376
24 Motley, A. M., Hettema, E. H., Hogenhout, E. M., Brites, P., ten Asbroek, A. L., Wijburg, F. A., Baas, F., Heijmans, H. S., Tabak, H. F., Wanders, R. J. and Distel, B. (1997) Nat. Genet. **15**, 377–380
25 Purdue, P. E., Zhang, J. W., Skoneczny, M. and Lazarow, P. B. (1997) Nat. Genet. **15**, 381–384
26 Elgersma, Y., Elgersma-Hooisma, M., Wenzel, T., McCaffery, J. M., Farquhar, M. G. and Subramani, S. (1998) J. Cell Biol. **140**, 807–820
27 Schumann, U., Gietl, C. and Schmid, M. (1999) Plant Physiol. **120**, 339
28 Rehling, P., Marzioch, M., Niesen, F., Wittke, E., Veenhuis, M. and Kunau, W.-H. (1996) EMBO J. **15**, 2901–2913
29 Zhang, J. W. and Lazarow, P. B. (1996) J. Cell Biol. **132**, 325–334
30 Rachubinski, R. A. and Subramani, S. (1995) Cell **83**, 525–528
31 Gould, S. J., Kalish, J. E., Morrell, J. C., Bjorkman, J., Urquhart, A. J. and Crane, D. I. (1996) J. Cell Biol. **135**, 85–95
32 Erdmann, R. and Blobel, G. (1996) J. Cell Biol. **135**, 111–121
33 Komori, M., Rasmussen, S. W., Kiel, J. A., Baerends, R. J., Cregg, J. M., van der Klei, I. J. and Veenhuis, M. (1997) EMBO J. **16**, 44–53
34 Albertini, M., Rehling, P., Erdmann, R., Girzalsky, W., Kiel, J. A., Veenhuis, M. and Kunau, W.-H. (1997) Cell **89**, 83–92
35 Brocard, C., Lametschwandtner, G., Koudelka, R. and Hartig, A. (1997) EMBO J. **16**, 5491–5500
36 Huhse, B., Rehling, P., Albertini, M., Blank, L., Meller, K. and Kunau, W.-H. (1998) J. Cell Biol. **140**, 49–60
37 Girzalsky, W., Rehling, P., Stein, K., Kipper, J., Blank, L., Kunau, W.-H. and Erdmann, R. (1999) J. Cell Biol. **144**, 1151–1162
38 Fransen, M., Terlecky, S. R. and Subramani, S. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 8087–8092
39 Will, G. K., Soukupova, M., Hong, X., Erdmann, K. S., Kiel, J. A., Dodt, G., Kunau, W.-H. and Erdmann, R. (1999) Mol. Cell. Biol. **19**, 2265–2277
40 Schliebs, W., Saidowsky, J., Agianian, B., Dodt, G., Herberg, F. W. and Kunau, W.-H. (1999) J. Biol. Chem. **274**, 5666–5673
41 Shimizu, N., Itoh, R., Hirono, Y., Otera, H., Ghaedi, K., Tateishi, K., Tamura, S., Okumoto, K., Harano, T., Mukai, S. and Fujiki, Y. (1999) J. Biol. Chem. **274**, 12593–12604
42 Braverman, N., Dodt, G., Gould, S. J. and Valle, D. (1998) Hum. Mol. Genet. **7**, 1195–1205
43 Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. and Hieter, P. (1992) Gene **110**, 119–122
44 Rothstein, R. (1991) Methods Enzymol. **194**, 281–301
45 Kozak, M. (1986) Cell **44**, 283–292
46 Burnette, W. N. (1981) Anal. Biochem. **112**, 195–203
47 Aitchison, J. D., Murray, W. W. and Rachubinski, R. A. (1991) J. Biol. Chem. **266**, 23197–23203
48 Aitchison, J. D., Szilard, R. K., Nuttley, W. M. and Rachubinski, R. A. (1992) Yeast **8**, 721–734
49 Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J. and Subramani, S. (1989) J. Cell Biol. **108**, 1657–1664
50 Lamb, J. R., Tugendreich, S. and Hieter, P. (1995) Trends Biochem. Sci. **20**, 257–259

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