Yarrowia lipolytica Pex20p, *Saccharomyces cerevisiae* Pex18p/Pex21p and mammalian Pex5pL fulfil a common function in the early steps of the peroxisomal PTS2 import pathway

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Import of peroxisomal matrix proteins is essential for peroxisome biogenesis. Genetic and biochemical studies using a variety of different model systems have led to the discovery of 23 *PEX* genes required for this process. Although it is generally believed that, in contrast to mitochondria and chloroplasts, translocation of proteins into peroxisomes involves a receptor cycle, there are reported differences of an evolutionary conservation of this cycle either with respect to the components or the steps involved in different organisms. We show here that the early steps of protein import into peroxisomes exhibit a greater similarity than was thought previously to be the case. Pex20p of *Yarrowia lipolytica*, Pex18p and Pex21p of *Saccharomyces cerevisiae* and mammalian Pex5pL fulfil a common function in the PTS2 pathway of their respective organisms. These non-orthologous proteins possess a conserved sequence region that most likely represents a common PTS2-receptor binding site and di-aromatic pentapeptide motifs that could be involved in binding of the putative docking proteins. We propose that not necessarily the same proteins but functional modules of them are conserved in the early steps of peroxisomal protein import.

INTRODUCTION

Peroxisomes are ubiquitous organelles of eukaryotic cells. The discovery of inherited peroxisomal diseases and the need for their molecular analysis has led to the use of genetically tractable model systems to dissect peroxisome biogenesis (Lazarow, 1993).

Currently, 23 *PEX* genes have been identified (http://www.mips.biochem.mpg.de/proj/yeast/reviews/pex_table.html). Their gene products are collectively called peroxins. Several of them have been found in such distantly related eukaryotic organisms as different yeast species, Chinese hamster ovary cells, human fibroblasts and, most recently, plants. These findings have led to the concept that the mechanisms of peroxisome biogenesis are conserved throughout evolution.

This belief has been strengthened by the fact that peroxisome protein import in most organisms shares characteristic features (for a review see Subramani *et al.*, 2000). Matrix protein import is mediated by two import pathways. Two ubiquitous targeting signals, PTS1 and PTS2, have been well characterized. These are recognized and bound by two predominantly cytosolic import receptors, Pex5p and Pex7p, respectively. One widely accepted model of their function involves a receptor cycle (Dodt and Gould, 1996; Kunau and Erdmann, 1998; Dammai and Subramani, 2001). The cargo proteins bind to the receptor in the cytosol, which facilitates binding to the membrane via docking proteins, followed by the import of the cargo protein into the peroxisome and return of the free receptor to the cytosol (receptor shuttle). There is increasing evidence to suggest strongly that the receptor travels at least into the peroxisomal membrane and perhaps even into the matrix before returning to the cytosol (extended shuttle) (Gouveia *et al.*, 2000; Dammai and Subramani, 2001). The most likely candidates among the peroxins for the putative docking proteins are Pex13p (Elgersma *et al.*, 1996; Erdmann and Blobel, 1996; Gould *et al.*, 1996) and Pex14p (Albertini *et al.*, 1997; Brocard *et al.*, 1997).

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Fig. 1. YIPex20p partially restores the oleic acid growth and thiolase import of the *S. cerevisiae* double mutant strain *pex18Δpex21Δ*. Growth on oleate plate (**A**) and in liquid oleate medium (**B**) was tested with the *pex18Δpex21Δ* strain transformed with the YIPEX20 expression plasmid. For the drop dilution assay, equal amounts of cells were diluted in distilled water and aliquots were applied onto oleic acid plates (spots on the left correspond to 2×10^5 cells). In liquid medium the growth rate of *pex18Δpex21Δ* cells expressing YIPex20p under the control of the PEX7 promoter is reduced at least 2-fold when compared to wild type. No growth at all was observed for the *pex18Δpex21Δ* strain on plate and in liquid medium. (**C**) Iodixanol equilibrium density centrifugation was performed with *S. cerevisiae* wild-type, *pex18Δpex21Δ* and *pex18Δpex21Δ* transformed with the YIPEX20 expression plasmid. Post-nuclear supernatants were obtained from cells grown on oleate for 14 h. All fractions were analysed for the activities of a mitochondrial (cytochrome C-oxidase) and a peroxisomal (catalase) enzyme (upper panel). Equal volumes of gradient fractions were analysed by western blot using antibodies against thiolase (lower panel).

However, there are also a number of distinct differences between the organisms that have been studied and which argue against a conserved mechanism for peroxisomal protein import. A number of PEX genes still have been found only in one or two organisms (http://www.mips.biochem.mpg.de/proj/yeast/reviews/pex_table.html). A very intriguing point is that different peroxins have been reported to be indispensable for the import of PTS2 proteins in the three organisms *Saccharomyces cerevisiae*, *Yarrowia lipolytica* and human cells. In *S. cerevisiae* two additional, predominantly cytosolic, proteins, Pex18p and Pex21p, are required besides the PTS2 receptor Pex7p to target the PTS2 protein thiolase into peroxisomes (Purdue et al., 1998). These two proteins are functionally redundant and bind to

Pex7p but not to thiolase. In contrast, in *Y. lipolytica* no Pex7p orthologue and neither one of the two putative docking proteins have yet been found. Instead, a new peroxin Pex20p has been reported to bind thiolase (Titorenko et al., 1998) and is inserted into the peroxisomal membrane (Smith and Rachubinski, 2001). Moreover, Pex20p has not been discovered in any other organism. The distinct features of thiolase import into peroxisomes of *Y. lipolytica* are of special concern. This is because one of the two current models of peroxisome biogenesis is based on unrelated observations that have been made only in this yeast species (Titorenko and Rachubinski, 2001). A third scenario for the PTS2 pathway has been documented in human cells. Here the import of PTS2 proteins is dependent not only on the PTS2

Fig. 2. YIPex20p expressed in *S. cerevisiae* interacts with the same proteins as ScPex21p in the two-hybrid assay. Deletions in PCY2 strains are indicated. Three independent colonies of each transformation were transferred on filter discs and assayed for β-galactosidase activity. Reactions were stopped after 1 or 12 h: top panel, 12 h; middle panel, 1 h; bottom panel, 1 h (above the dashed line) and 12 h (below the dashed line).

receptor Pex7p, but also on a long version of the PTS1 receptor Pex5p (Braverman *et al.*, 1998; Otera *et al.*, 1998). In mammals two different forms of the PTS1 receptor are known, Pex5pS and Pex5pL (Dodt *et al.*, 1995). These differ by only 37 amino acid residues, which are encoded by an additional exon.

In the light of these differences, we set out to explore what the peroxins ScPex18p, ScPex21p, YIPex20p and HsPex5pL might have in common, and which make them indispensable for the import of PTS2 proteins in their respective organisms.

RESULTS AND DISCUSSION

We first heterologously expressed YIPex20p in the double knock-out strain *pex18Δpex21Δ* of *S. cerevisiae* and found that it can functionally substitute for the two peroxins of *S. cerevisiae*. Both processes, growth on oleic acid (Figure 1A and B) as well as thiolase import into peroxisomes (Figure 1C), were abolished in the *pex18Δpex21Δ* strain but partially restored by the expression of YIPex20p. The results argue strongly that YIPex20p has a function in *Y. lipolytica* similar to that of Pex18p and Pex21p in *S. cerevisiae*. As the latter two peroxins exert their function in *S. cerevisiae* in association with Pex7p (Purdue *et al.*, 1998), an important implication is that *Y. lipolytica* also possesses an as yet unidentified PTS2 receptor Pex7p. This assumption is supported by homology searches in databases of ongoing *Neurospora crassa* genome projects (www.mips.biochem.mpg.de/proj/neurospora/ and www.genome.ou.edu/

Fig. 3. Sequence similarities among Pex20p, Pex18/Pex21p and Pex5p. (A) Sequence alignment of the putative Pex7p binding boxes. Sequences were aligned with the Clustal_W algorithm using the blosum62 matrix. In the human Pex5p sequence the region corresponding to the additional exon is underlined. The asterisk marks the position of the point mutation in mammalian Pex5 (S214F) that disrupts the interaction with Pex7p (Matsumura *et al.*, 2000). The *N. crassa* PEX20 homologue (b2c22.tfa_10wg) was identified from the MIPS *N. crassa* database by PEDANT. (B) Schematic representation of conserved sequence regions. The number of WxxxF-motifs [WF] is indicated by the subscript numbers. Small boxes represent the putative Pex7p binding box. The large boxes in the C-terminal halves of Pex5 proteins correspond to the TPR motifs.

fungal.html), indicating that this filamentous fungus also possesses homologues Pex20p and Pex7p.

We analyzed next the observed functional complementation in more detail by investigating possible interactions of YIPex20p with the *S. cerevisiae* proteins thiolase, Pex7p and the two putative docking proteins Pex13p and Pex14p. Using the two-hybrid system we obtained qualitatively identical results for YIPex20p and ScPex21p (Figure 2). Both peroxins interacted with thiolase and Pex7p, but in the case of thiolase only in the presence of Pex7p. Novel binding partners for YIPex20p and ScPex21p were identified by the interaction of both proteins with Pex13p (Figure 2) and Pex14p (data not shown). The interaction with Pex14p was, however, very weak and could be enhanced in the absence of Pex13p. Interestingly, the interaction of YIPex20p as well as of ScPex21p with Pex13p was independent of the presence of Pex7p. These results suggest that *Y. lipolytica* could have a PTS2 import pathway similar to that of *S. cerevisiae* with the import receptor Pex7p, the putative docking proteins Pex13p/Pex14p and YIPex20p serving the same role as Pex18p/Pex21p in *S. cerevisiae*.

How can the non-orthologous peroxins ScPex18p/Pex21p and YIPex20p fulfil the same function in their respective organisms? We looked into this question by aligning their sequences and

Fig. 4. Mutagenesis of a conserved serine within the putative Pex7p binding boxes of YIPex20p, ScPex18p and ScPex21p disrupts the two-hybrid interaction with ScPex7p. Double transformants of the PCY2 strain were transferred to filter discs. β -galactosidase staining was performed for 1 h.

found that they possess a common conserved region at their C-terminal ends (Figure 3A). The regions of ScPex18p/21p are part of larger fragments reported previously to interact with Pex7p (Purdue *et al.*, 1998). This conserved stretch of amino acid residues also aligned exactly with a short sequence region within the N-terminal half of HsPex5pL. Part of this region overlaps with those amino acids that distinguish the long and short form of HsPex5p and are essential for targeting of PTS2 proteins (Braverman *et al.*, 1998; Otera *et al.*, 1998). Very recently, Dodt *et al.* (2001) reported that the human Pex5pL fragment (amino acids 191–222) overlapping with the stretch of conserved amino acids (Figure 3) is sufficient for binding of HsPex7p. Moreover, a larger fragment (amino acids 191–251) comprising the complete region gives the strongest two-hybrid interactions. Furthermore, a missense mutation (S280F) within the relevant region was shown to disrupt the interaction between mammalian Pex5pL and Pex7p (Matsumura *et al.*, 2000). The introduction of the corresponding mutations into YIPex20p, ScPex18p and ScPex21p abolished the interaction with ScPex7p (Figure 4). These results document that the serine residue in a central position of the conserved region of all five peroxins of Figure 3 is critical for the interaction with Pex7p. Taken together, these data suggest strongly that the conserved region shown in Figure 3A represents a Pex7p binding box. However, it remains to be established that this interaction is a direct one. The fact that this sequence motif is not present in fungal Pex5p orthologues is in line with the notion that the fungal peroxins Pex18p, Pex20p and Pex21p take over a function similar to that served by Pex5pL in mammals, i.e. to assist Pex7p in the import of PTS2 proteins.

Another common structural feature, the WXXXF motif, is shared by YIPex20p, ScPex18p/Pex21p and mammalian Pex5pL. All known PTS1 receptors possess several repeats of this motif (Schliebs *et al.*, 1999). Seven of them were recently identified as high-affinity binding sites for HsPex14p in HsPex5pL (Saidowsky *et al.*, 2001). In ScPex5p one of the two WXXXF motifs is part of a larger Pex13p binding site (Bottger *et al.*, 2000). The existence of this motif in YIPex20p and ScPex18p/Pex21p could provide a molecular explanation as to why all these peroxins are capable of binding to Pex13p and/or Pex14p.

Taken together, these results indicate that the first steps of the peroxisomal protein import are more conserved than the

involvement of the non-orthologous peroxins YIPex20p, ScPex18p/Pex21p and HsPex5pL would seem to suggest.

METHODS

Strains and growth conditions. *Saccharomyces cerevisiae* strains used for complementation analysis were wild-type strain UTL7A (*MAT α* , *ura3-52*, *trp1*, *leu2-3,112*) (W. Duntze, Bochum, Germany) and its derivative *pex18 Δ pex21 Δ* . *Saccharomyces cerevisiae* strains used for two-hybrid analysis were PCY2 (*MAT α* , *gal4 Δ* , *gal80 Δ* , *URA3::GAL1-lacZ*, *lys2-801^{amber}*, *his3- Δ 200*, *trp1- Δ 63*, *leu2 ade2-101^{ochre}*) (Chevray and Nathans, 1992) and its derivatives *pex7 Δ* and *pex18 Δ pex21 Δ* . Deletion mutants were constructed using the kanMX-Marker (Güldener *et al.*, 1996).

Complete and minimal media used for yeast culturing have been described previously (Erdmann *et al.*, 1989). YNO medium contained 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0. To obtain the growth curves, cells were precultured to mid-log phase in 0.3% glucose SD medium and then transferred to liquid YNO medium. Manipulation of yeast cells was performed according to standard methods.

Vectors. The YIPEX20 open reading frame (ORF) was amplified by PCR using *Y. lipolytica* genomic DNA as template and subcloned using PCR-generated restriction sites into *EcoRI*–*SalI*-digested pYADE4, resulting in pHE20/2. A 1.6 kb fragment containing the YIPEX20 ORF and the CYC1 termination was excised from plasmid pHE20/2 and subcloned into *EcoRI*–*HindIII*-digested pRS416, resulting in pHE20/3. Finally, a 444 bp upstream region of the ScPEX7 ORF was amplified from *S. cerevisiae* genomic DNA by PCR introducing a 5' *SpeI* site and a 3' *SmaI* site and then subcloned into *SpeI*–*SmaI*-digested pHE20/3, resulting in pHE20/4. DNA sequence analysis from two independent PCR amplifications revealed a sequence conflict when compared to the original sequence (DDBJ/EMBL/GenBank accession No. AF054613). According to our analysis, the nucleotides CG at position 1210 are replaced by the nucleotides GGC resulting in the new C-terminal sequence GVAKQSNWEEDYDF (amino acids 404 to 417).

To obtain the two-hybrid vectors pPC86–ScPEX21, pPC86–YIPEX20, pPC97–ScFOX3 and pPC97–ScPEX13, the ORFs were amplified by PCR using the respective wild-type genomic DNA as templates. PCR-introduced 5' *BglII* and 3' *SalI* sites were used to subclone the fragments into pPC86 or pPC97, respectively. The point mutants YIPex20p(S280F), ScPex18p(S230F) and ScPex21p(S234F) were generated by overlap extension PCR (Higuchi *et al.*, 1988). DNA sequencing control revealed an additional point mutation in the sequence of PEX18(S230F), resulting in the substitution of the non-conserved amino acid serine 238 with an asparagine. pPC97–ScPEX7 has been described previously (Rehling *et al.*, 1996).

Density gradient centrifugation. Organelle preparation by differential centrifugation of yeast lysates was performed as described (Erdmann *et al.*, 1989). For separation of cell organelles, post-nuclear supernatants each containing 6 mg of protein were loaded onto continuous 15.5–36% (w/v) iodixanol gradients (25 ml) and then centrifuged for 90 min in a SV288 rotor at 19 500 r.p.m.

Anti-thiolase (Fox3p) antibodies have been described previously (Erdmann, 1994).

Two-hybrid analysis. The two-hybrid assay was based on the method of Fields and Song (1989). Transformants were streaked out on filter disks (Whatman Inc., Clifton, NJ), which were placed on SD synthetic medium plates lacking tryptophan and leucine and incubated overnight at 30°C, the cells grown in and on the disks were lysed by freezing in liquid N₂. β -galactosidase filter assays were then performed as described (Rehling *et al.*, 1996).

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