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Pex14p is a member of the protein linkage map of Pex5p

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To identify members of the translocation machinery for peroxisomal proteins, we made use of the two-hybrid system to establish a protein linkage map centered around Pex5p from *Saccharomyces cerevisiae*, the receptor for the C-terminal peroxisomal targeting signal (PTS1). Among the five interaction partners identified, Pex14p was found to be induced under conditions allowing peroxisome proliferation. Deletion of the corresponding gene resulted in the inability of yeast cells to grow on oleate as well as the absence of peroxisomal structures. The *PEX14* gene product of ~38 kDa was biochemically and ultrastructurally demonstrated to be a peroxisomal membrane protein, despite the lack of a membrane-spanning domain. This protein was shown to interact with itself, with Pex13p and with both PTS receptors, Pex5p and Pex7p, indicating a central function for the import of peroxisomal matrix proteins, either as a docking protein or as a releasing factor at the organellar membrane.

Keywords: peroxisomes/Pex5p/Pex14p/protein linkage map/two-hybrid system

Introduction

Proteins residing in the peroxisomal matrix are encoded in the nucleus, synthesized in the cytoplasm and imported post-translationally (Lazarow and Fujiki, 1985). Correct intracellular targeting is essential for life, as demonstrated by severe human diseases caused by mislocalization of usually peroxisomal proteins (Lazarow and Moser, 1989). The process of import is initiated through the recognition of organellar targeting signals by specific receptor proteins. So far, two different peroxisomal targeting signals have been identified: a C-terminal peroxisomal targeting signal 1 or PTS1 present in the majority of peroxisomal matrix proteins and first identified in luciferase (Gould *et al.*, 1987; for review, see Subramani, 1993) and a PTS2 usually located within the first 30 amino acids of a peroxisomal protein (Gietl, 1990; Swinkels *et al.*, 1991; for review, see deHoop and Ab, 1992). PTS1 consists of a tripeptide located at the C-terminus and characterized by the sequence SKL (or conservative variants), whereas PTS2 fits a loose consensus sequence, (R/K)(L/V/I)X_5(Q/H)(L/A) (Subramani, 1996). While each of the signals is sufficient for transport into peroxisomes, proteins may enter these organelles even if they lack a PTS provided that they are able to oligomerize with PTS-containing polypeptides (Glover *et al.*, 1994; McNew and Goodman, 1994). Peroxisomal membrane proteins are targeted to the organelle via the use of different targeting signals defined only recently (Subramani, 1996).

In the yeast *Saccharomyces cerevisiae*, mutants unable to use oleate as carbon source have been found (Erdmann *et al.*, 1989; van der Leij *et al.*, 1992; Elgersma *et al.*, 1993). These carried defects in structural genes for enzymes in the β-oxidation pathway (*fox* mutants) or defects in genes encoding peroxins (*pex* mutants, Distel *et al.*, 1996) characterized by the mislocalization of peroxisomal proteins to the cytosol. Two gene products, Pex5p and Pex7p, were shown to function as receptors for the two peroxisomal targeting signals. In *pex7* yeast cells, thiolase was excluded from the peroxisomal compartment, whereas PTS1-containing proteins were imported correctly (Marzioch *et al.*, 1994; Zhang and Lazarow, 1995). In addition, Pex7p interacts specifically with the PTS2 of thiolase (Rehling *et al.*, 1996), corroborating the evidence that *PEX7* encodes the receptor for the PTS2. In cells deleted at *PEX5*, PTS1-containing proteins accumulated in the cytosol (Van der Leij *et al.*, 1993). In the two-hybrid system, the tetratricopeptide repeat (TPR) domain of Pex5p interacts with the C-terminal SKL (Brocard *et al.*, 1994). The human homolog was cloned by a two-hybrid screening of a human liver cDNA library and was demonstrated to bind to PTS1 peptides *in vitro* (Fransen *et al.*, 1995), like the homolog from *Pichia pastoris* (McCollum *et al.*, 1993), strengthening the evidence that these gene products are indeed receptors for the PTS1 signals. Recent results from localization studies demonstrate its predominantly cytoplasmic location (Elgersma *et al.*, 1996; Gould *et al.*, 1996), suggesting that Pex5p acts as a mediator between proteins targeted to peroxisomes and the organelle itself. This implies that in addition to binding to PTS1, Pex5p may carry peroxisomal proteins towards their destination by interacting with other factors either involved in import of peroxisomal proteins or located in the membrane. Indeed, a docking protein for Pex5p in the peroxisomal membrane, termed Pex13p, was identified by three groups (Erdmann and Blobel, 1996; Elgersma *et al.*, 1996; Gould *et al.*, 1996). Taken together, the products of the three genes *PEX5*, *PEX7* and *PEX13* have been shown to be involved directly in the peroxisomal import process, though the peroxisomal translocation machinery remains the least understood part of the peroxisomal import system. To gain more information about the components of the peroxisomal import machinery, we searched for possible

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-492              AATGGAAGAAAACGATTGTTGTCTAAGAATGGCTTACCGAAGCTGCCCTTG
-441  TCAAATTTCAGCTCTTGAAAATTTAAGCCCGTTACTATAGAGCAGCCAAACAACTGCAGCAGC
-378  GGCTGCATAGATTTGAACATCTATCGTGAAACGATTTTTTATTGAGGATTCTGGCTTGAGACG
-315  CCAATGCAAAGTTCTCATTAATGCCTCGAACGTAAACTCATCCGCGAGTATATCCTCTTGAAT
-252  TTCAAAACGAATATACCTGCCCATGGTCTTACACCTGCCACCTTTGAAACTTCGCTTACTACT
-189  TCAGCGTTTTTAACCATCCACGGTTTTTTTGCTGAGTGATTCTCTTTCTCCTCATTCTCATTTT
-126  AGTATAGCGGTTTTAATAAGCGCCCGAAAGATAATTGTAAAACATATATTCAATGCTTAAAAA
 -63  TAAAGAAATTGCCCATCAATTTGAAAACTCAAGTAAAACAGAGAAGTTGTAAGGTGAATAAGG
   1  ATG AGT GAC GTG GTC AGT AAA GAT CGT AAG GCA TTG TTC GAC TCA GCA
        M   S   D   V   V   S   K   D   R   K   A   L   F   D   S   A   16
  49  GTA TCC TTT TTA AAG GAT GAG TCC ATT AAA GAT GCT CCA CTT TTA AAG
        V   S   F   L   K   D   E   S   I   K   D   A   P   L   L   K   32
  97  AAA ATC GAA TTT TTA AAA TCC AAA GGG TTA ACA GAA AAG GAG ATT GAA
        K   I   E   F   L   K   S   K   G   L   T   E   K   E   I   E   48
 145  ATA GCC ATG AAA GAG CCC AAG AAA GAC GGT ATC GTA GGC GAT GAA GTA
        I   A   M   K   E   P   K   K   D   G   I   V   G   D   E   V   64
 193  TCG AAA AAA ATT GGT AGT ACT GAG AAT AGA GCC TCA CAG GAT ATG TAT
        S   K   K   I   G   S   T   E   N   R   A   S   Q   D   M   Y   80
 241  CTC TAT GAA GCG ATG CCA CCA ACG CTG CCC CAC AGG GAT TGG AAG GAC
        L   Y   E   A   M   P   P   T   L   P   H   R   D   W   K   D   96
 289  TAT TTT GTG ATG GCT ACT GCC ACA GCT GGG CTG TTG TAT GGT GCA TAT
        Y   F   V   M   A   T   A   T   A   G   L   L   Y   G   A   Y  112
 337  GAA GTA ACT AGA AGG TAT GTG ATA CCA AAT ATT TTA CCA GAA GCA AAA
        E   V   T   R   R   Y   V   I   P   N   I   L   P   E   A   K  128
 385  AGC AAG TTG GAA GGG GAC AAA AAA GAA ATT GAT GAT CAG TTC TCC AAA
        S   K   L   E   G   D   K   K   E   I   D   D   Q   F   S   K  144
 433  ATC GAT ACA GTC CTC AAT GCC ATC GAA GCG GAG CAA GCT GAG TTT AGG
        I   D   T   V   L   N   A   I   E   A   E   Q   A   E   F   R  160
 481  AAA AAG GAA AGC GAA ACA TTA AAG GAA CTT AGT GAC ACG ATT GCT GAA
        K   K   E   S   E   T   L   K   E   L   S   D   T   I   A   E  176
 529  CTG AAA CAG GCG CTT GTG CAG ACA ACA AGA AGC AGG GAA AAG ATC GAA
        L   K   Q   A   L   V   Q   T   T   R   S   R   E   K   I   E  192
 577  GAC GAG TTT AGA ATA GTT AAA CTC GAG GTG GTC AAT ATG CAA AAT ACG
        D   E   F   R   I   V   K   L   E   V   V   N   M   Q   N   T  208
 625  ATC GAC AAA TTT GTT TCA GAT AAT GAC GGC ATG CAA GAG TTA AAT AAT
        I   D   K   F   V   S   D   N   D   G   M   Q   E   L   N   N  224
 673  ATC CAA AAA GAA ATG GAA TCT CTG AAA AGC TTA ATG AAT AAC CGT ATG
        I   Q   K   E   M   E   S   L   K   S   L   M   N   N   R   M  240
 721  GAA TCC GGT AAT GCG CAG GAC AAC AGA TTA TTT TCC ATA TCT CCT AAT
        E   S   G   N   A   Q   D   N   R   L   F   S   I   S   P   N  256
 769  GGT ATA CCT GGC ATA GAT ACG ATT CCA TCT GCG TCT GAG ATT CTT GCC
        G   I   P   G   I   D   T   I   P   S   A   S   E   I   L   A  272
 817  AAA ATG GGC ATG CAA GAA GAA AGT GAT AAA GAA AAG GAA AAC GGC AGC
        K   M   G   M   Q   E   E   S   D   K   E   K   E   N   G   S  288
 865  GAT GCT AAT AAA GAT GAC AAT GCT GTT CCA GCG TGG AAA AAA GCA AGA
        D   A   N   K   D   D   N   A   V   P   A   W   K   K   A   R  304
 913  GAA CAA ACT ATT GAT AGC AAC GCC TCC ATT CCA GAA TGG CAA AAA AAT
        E   Q   T   I   D   S   N   A   S   I   P   E   W   Q   K   N  320
 961  ACC GCC GCC AAT GAG ATC AGT GTC CCT GAC TGG CAA AAT GGA CAG GTC
        T   A   A   N   E   I   S   V   P   D   W   Q   N   G   Q   V  336
1009  GAA GAC TCC ATC CCA TAG CGC AAT TCT ATG TAA GTA ATT AGT TTT TTA
        E   D   S   I   P   *                                          341
1057  ACGGAAATTGTAATTGTAACTGTCCTAGATTACTATGGACATTCAATCTGATTGTTACCCGGA
1120  ACATTTGCTTTTGAAAGGCATTTTTAGTAGCACTAATATATTGGCAGCTAATTGAAACAGTAA
1183  TCTACAAATATGAGTAAGCTAAACCACCTCAACAAAGCTATTCGTGGCTAGTATATGACCTTG
1246  AATTGCCATGGAAAAAGAATCAGTATACAACTTAGCACTAAAATGTGCTGAGCGGCAATTGAC
1309  CTCCATGGAATTTTCGAACCTTTACAAGGAATTCTTTAATGAAAAGTTCCCGTCTTT
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Fig. 1. Nucleotide and deduced amino acid sequence of the *PEX14* gene. A putative oleate response element (Einerhand *et al.*, 1993; Filipits *et al.*, 1993) and a presumptive TATA box (Guarante, 1992) in the 5′ non-coding region are underlined. A proline-rich sequence motif (Feng *et al.*, 1994) is indicated by bold italics and the predicted coiled-coil region (Lupas *et al.*, 1991) of the polypeptide is underlined.

interacting partners of Pex5p and we established its protein linkage map (Evangelista *et al.*, 1996) using the two-hybrid system (Fields and Song, 1989).

Here we report the identification and characterization of a new protein involved in peroxisomal assembly in *S.cerevisiae*. This protein was identified in a two-hybrid screen as a factor interacting with Pex5p, and sequence comparison revealed homology of this gene with *PEX14* from *Hansenula polymorpha* (Komori *et al.*, 1997). The gene encodes a protein of 341 amino acids with an estimated mol. wt of 38 kDa. The inability of cells carrying a deletion of this gene to grow on oleate, the lack of peroxisomes in these mutant cells and the interactions of Pex14p with at least three other peroxins indicate the involvement of this protein in peroxisome biogenesis.

Results

Pex14p interacts with Pex5p

We employed the two-hybrid system to search for interaction partners of the Pex5 protein. Yeast strain HF7c expressing a Gal4-binding domain–Pex5 protein fusion (pGBT9-*Sc*Pex5p) was transformed with a genomic yeast library encoding fusion proteins between the Gal4 activation domain and random genomic fragments (James *et al.*, 1996). A total of 5×10^6 transformants harboring both plasmids were tested. Among those, 216 candidates were able to grow on media without histidine in the presence of 20 mM 3-amino-1,2,4-triazol (AT). Of the 216 colonies, 34 turned blue using β-galactosidase filter assays. Plasmid isolation, retransformation and subsequent analysis for growth without histidine in the presence of 20, 35 or 55 mM AT as well as β-galactosidase filter assays resulted in the identification of seven plasmids expressing proteins that interacted specifically with Pex5p. Following bidirectional sequencing of the inserts using oligonucleotides H278 and H279, three plasmids were found to harbor the same open reading frame (ORF) (YGL153w) fused at the same codon. The five different sequences were compared with the complete *S.cerevisiae* genome sequence, but none of the corresponding genes has yet been characterized. We chose the sequence represented in our screen three times, and subsequently shown to be similar to the *H.polymorpha PEX14* gene (Komori *et al.*, 1997), for further characterization.

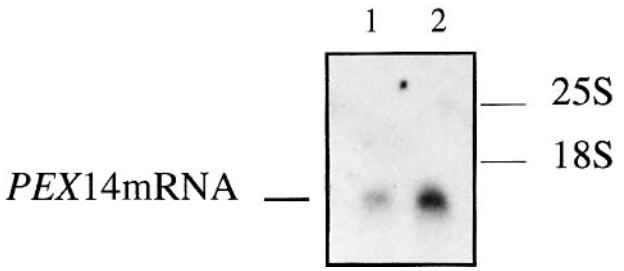

Fig. 2. Northern blot analysis of *PEX14* mRNA produced in wild-type cells (CB80). Cells were grown on medium containing glucose (lane1) or oleate (lane 2), and 20 µg of total RNA was loaded in each lane. The 1.1 kb *Sca*I–*Eco*RI fragment of *PEX14* (bases 208–1337 in Figure 1) was used as a probe for hybridization.

Sequence and expression of PEX14

The sequence of *PEX14* is depicted in Figure 1 (GenBank accession No. Z72675 and EMBL Z48618). The promoter sequence (–492 to –1) does not contain known transcriptional control elements other than a pair of palindromic CGG triplets with an 11 bp interval (underlined with dots in Figure 1) that resembles an oleate response element (ORE, Einerhand *et al.*, 1993; Filipits *et al.*, 1993) but with shorter spacing (Rottensteiner *et al.*, 1996). The *PEX14* gene encodes a polypeptide with a calculated mass of 38.4 kDa. The deduced amino acid sequence of 341 residues contains putative glycosylation sites, a number of possible phosphorylation sites (not indicated) and the proline-rich motif PXXP known to allow interactions with SH3 domains of other proteins (Feng *et al.*, 1994). In addition, Pex14p contains a predicted coiled-coil region (underlined in Figure 1; Lupas *et al.*, 1991) similar to kinesin-related proteins, but no known targeting signal which would indicate a distinct intracellular location. Other than 35% identity and 56% similarity to *Hp*Pex14p, no similarity to any other protein in the databases was found. The inserts of the original plasmids from the library encoded the protein from the fourth amino acid to the end, with different lengths of 3′-non-coding regions. Northern blot analysis revealed a low constitutive expression of a single transcript from this gene (~1.2 kb) on glucose, and a 5-fold induction on oleate (Figure 2).

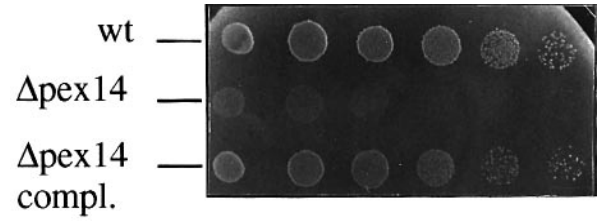

Fig. 3. Functional complementation of Δ*pex14* cells in terms of growth on oleate medium. Wild-type cells CB80 (wt), mutant cells CB81 (Δ*pex14*) and mutant cells containing the plasmid YCp*PEX14* were grown on plates containing 0.2% oleate as the sole carbon source. Ten-fold dilutions were made, and 3 μl of each were applied directly onto the surface of the plates. The plates were incubated at 30°C for 6 days and photographed.

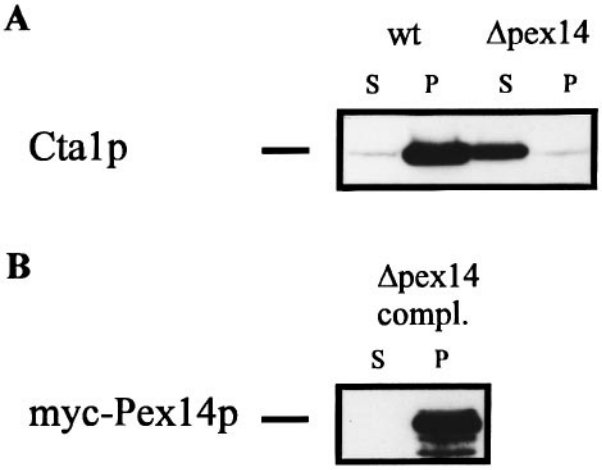

Fig. 4. Western blots demonstrating the distribution of catalase A (**A**) and Pex14p (**B**) in the post-organellar supernatant (S) and the organellar pellet (P) fraction. Catalase A is found in the organellar pellet from wild-type cells and in the post-organellar supernatant from Δ*pex14* mutant cells. Pex14p is found in the organellar pellet fraction from Δ*pex14* mutant cells expressing the myc-tagged Pex14p from YCp-myc-Pex14 (B). Equal amounts of protein derived from wild-type (wt), mutant cells (Δ*pex14*) or mutant cells complemented by YCp-myc-Pex14 (Δ*pex14* compl.) were separated by SDS–PAGE and analyzed by Western blot using antibodies against catalase A (A) or against the myc epitope (B).

Cells deleted at PEX14 do not contain peroxisomes

The genomic copy of *PEX14* was disrupted in strain FY1679 (Winston *et al.*, 1995) using the *Kan*MX module from plasmid pFA6a-*Kan*MX4 (Wach *et al.*, 1994). The growth phenotype of cells from the descendant haploid mutant strain CB81 was compared with the growth behavior of isogenic wild-type cells (CB80). On media containing glucose, ethanol, acetate or glycerol as carbon source, no difference was detected (not shown) but, in contrast to the wild-type cells, the mutant cells were unable to grow on oleate. Transformation with YCp*PEX14* restored the growth of the mutant cells on oleate (Figure 3). Catalase A, normally located in peroxisomes, was detected in the organellar pellet from induced wild-type cells, whereas the majority of this protein was found in the post-organellar supernatant from mutant cells grown under the same conditions (Figure 4A), indicating the lack of functional peroxisomes in the mutant cells. Electron microscopy (Figure 5A and B) demonstrated the absence of peroxisomes from mutant cells, therefore defining

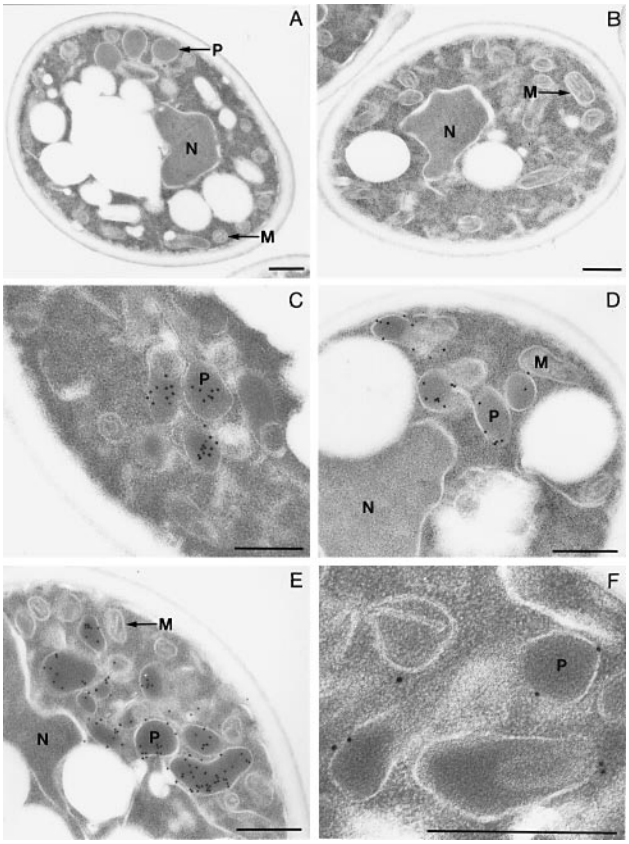

Fig. 5. Electron microscopy (A and B) and immunoelectron microscopy (C–F) of wild-type cells (**A** and **D**), wild-type cells expressing GFP-SKL (**C**), Δ*pex14* mutant cells (**B**) and Δ*pex14* mutant cells expressing myc-tagged Pex14p (**E** and **F**) from plasmid YCp-myc-Pex14. In contrast to the wild-type (A), no peroxisomal profiles could be detected in mutant cells (B). The PTS1-containing GFP is imported into peroxisomes of wild-type cells (C) as well as thiolase, a PTS2-type protein (D). The tagged Pex14p restores import of thiolase into peroxisomes (compare E and D), and is detected in peroxisomal membranes (F). Ultrathin sections were probed with polyclonal antiserum against thiolase (1:1000 diluted, D and E) or against GFP (1:150 diluted, C) followed by incubation with protein A conjugated with 14 nm gold (1:50 diluted), or with undiluted hybridoma supernatant (9E10) followed by incubation with goat anti-mouse IgG coupled to 10 nm gold (diluted 1:50). Bars represent 0.5 μm; M, N and P indicate mitochondria, nuclei and peroxisomes, respectively.

Pex14p as a peroxin (Distel *et al.*, 1996). In addition, wild-type and mutant cells were transformed with plasmid pJR233 encoding the green fluorescent protein (GFP) from *Aequorea victoria* (Prasher *et al.*, 1992) with a PTS1 at the C-terminus (Monosov *et al.*, 1996) under the control of the *MLS1* promoter (Hartig *et al.*, 1992). After induction of peroxisomes on oleate, wild-type cells showed a punctate staining pattern using fluorescence microscopy, whereas in mutant cells this reporter protein fluoresced evenly within the cells (Figure 6A and B). Immunofluorescence using antibodies against thiolase, a PTS2-carrying peroxisomal matrix protein, on wild-type and mutant cells resulted in a similar staining pattern (Figure 6C and D). This punctate appearance in wild-type cells is due to the peroxisomal location of both proteins demonstrated by qualitatively indistinguishable decoration of peroxisomes with antibodies against GFP (Figure 5C) and against thiolase (Figure 5D) using electron microscopy.

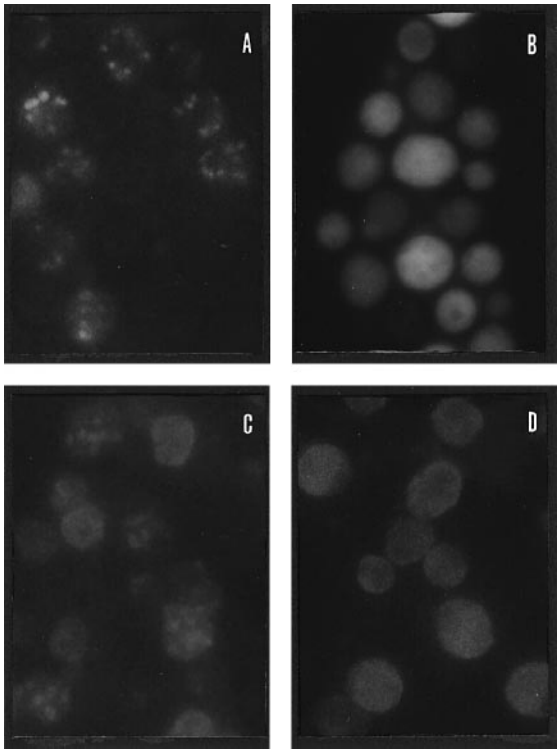

Fig. 6. Fluorescence of wild-type (**A**) and Δ*pex14* mutant cells (**B**) expressing GFP–SKL from plasmid pJR233, and immunofluorescence of wild-type (**C**) and Δ*pex14* mutant cells (**D**) using antibodies against thiolase. Transformed and non-transformed wild-type (CB80) and mutant cells (CB81) were cultured on oleate and inspected under the fluorescence microscope with (C and D) or without (A and B) prior processing for immunofluorescence.

A myc-tagged version of Pex14p expressed under the control of the native promoter using a centromeric plasmid (YCp-myc-Pex14) restored the ability of the mutant cells both to grow on oleate and to induce peroxisomes (Figure 5E), and thus served as a useful tool in subsequent localization studies.

Pex14p is a peroxisomal membrane protein

Subcellular fractionation of oleate-induced Δ*pex14* cells complemented with the myc-tagged Pex14p resulted in its presence in the organellar pellet (Figure 4B). From this pellet, peroxisomes were purified and extracted with potassium chloride and with sodium carbonate (Erdmann and Blobel, 1995). The tagged Pex14p was found predominantly in the membrane pellet (Figure 7). In addition, peroxisomal membranes were decorated with anti-myc antibodies using immunoelectron microscopy (Figure 5F). Together, these results demonstrated that Pex14p is either an integral membrane- or a tightly membrane-associated protein. Purified peroxisomes from oleate-induced Δ*pex14* cells complemented with the myc-tagged Pex14p were incubated with different concentrations of trypsin (Figure 8). Whereas the peroxisomal matrix protein catalase A was completely protected, the antigenic parts of myc-Pex14p and Pex13p were degraded by the protease added. Therefore, the N-terminus of myc-Pex14p faces the cytosolic side of the peroxisomal membrane like the SH3 domain of Pex13p (Elgersma *et al.*, 1996).

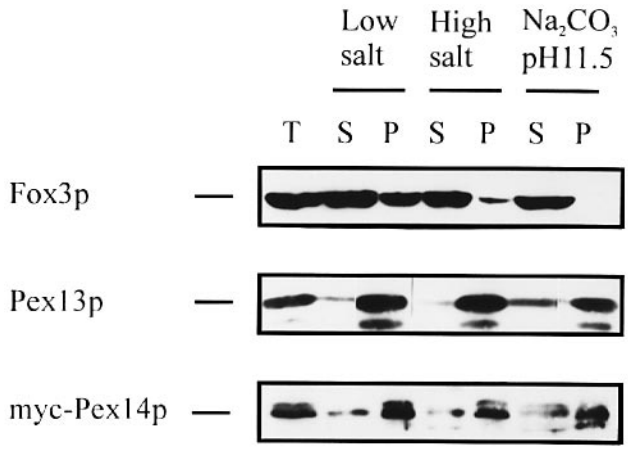

Fig. 7. Pex14p is a peroxisomal membrane protein. Peroxisomes from strain CB81 (Δ*pex14*) expressing the myc-tagged Pex14p were purified by density gradient centrifugation and extracted by low salt (LS), high salt (HS) or sodium carbonate at pH 11.5 as indicated. Equivalent amounts of total starting material (T), and of supernatant (S) and pellet (P) fractions of a 100 000 *g* sedimentation after each extraction were precipitated by TCA and analyzed by immunoblotting using either polyclonal antibodies or 9E10 hybridoma supernatant to detect thiolase (Fox3p), Pex13p and myc-tagged Pex14p, respectively.

Pex14p interacts with components of the peroxisomal translocation machinery

The interaction between Pex5p and Pex14p was demonstrated independently by co-immunoprecipitation. Myc antibodies were applied to total protein extracts from cells deleted for *PEX14* and *PEX5* (CB83) and transformed with plasmids encoding the myc-tagged version of Pex14p (YCp-myc-Pex14) and the hemagglutinin (HA)-tagged version of Pex5p (YCp-Pex5-HA). The presence of Pex5p-HA was demonstrated in the immunoprecipitate (Figure 9).

Semiquantitative filter assays for β-galactosidase activities using 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) were applied to determine interactions between Pex14p and proteins known to be substrates or components of the peroxisomal import machinery (see Table I). Strong two-hybrid interactions were found between Pex14p and both receptors, Pex5p and Pex7p. In the same system, Pex14p was shown to interact with another peroxisomal membrane protein, Pex13p, that in turn was demonstrated to be the docking protein for Pex5p (Elgersma *et al.*, 1996; Erdmann and Blobel, 1996; Gould *et al.*, 1996). Interestingly, Pex14p is also capable of interacting with itself, suggesting that it acts as an oligomer. However, no interaction was found between Pex14p and the PTS1-containing protein previously shown to be imported efficiently into peroxisomes *in vivo* (subunit IV of cytochrome *c*-oxidase extended by PTS1; Kragler *et al.*, 1993).

Discussion

To identify components of the peroxisomal translocation machinery, we employed the two-hybrid system following the idea of establishing a protein linkage map (Bartel *et al.*, 1996; Evangelista *et al.*, 1996) as a network of interactions centered around Pex5p. Seven plasmids were found harboring five different inserts reported as ORFs of unknown function in the *S.cerevisiae* genome. Among the five different clones found, one contained an ORF that

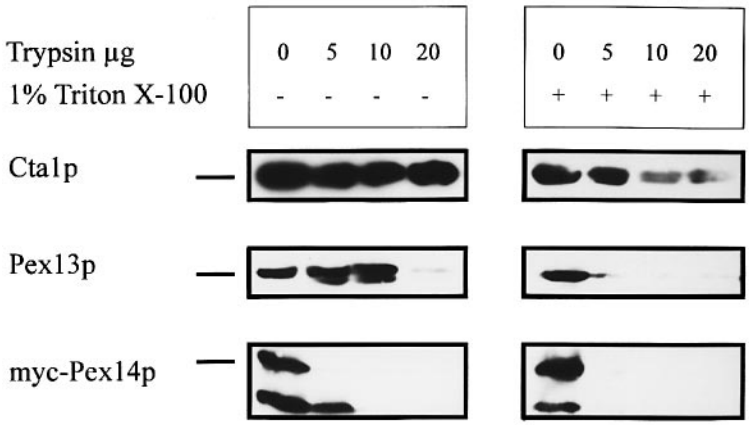

Fig. 8. The N-terminus of Pex14p faces the cytosol. An organellar fraction from the oleate-cultured strain CB81 (Δ*pex14*) expressing the myc epitope-tagged Pex14p was subjected to trypsin digestion in the absence or presence of detergent as indicated. Degradation of catalase (Cta1p), Pex13p and myc-Pex14p was followed by immunoblotting.

encodes a WD40 protein (but not Pex7p) and another encoded a putative integral membrane protein of no known function. We chose for further characterization the insert represented three times in the screen, since the corresponding gene was known to contain a DNA sequence that resembles an ORE (Einerhand *et al.*, 1993; Filipits *et al.*, 1993).

The two-hybrid screen was performed using a bait with clearly detectable self-activation. Therefore, suppression of the background activation of the *HIS3* reporter was essential for identification of interacting plasmids, as was the concentration of AT. Plasmids chosen for further characterization allowed growth on plates without histidine even in the presence of 55 mM AT, and the specificity of interaction was demonstrated by the development of blue color using X-Gal. The interactions that gave rise to β-galactosidase activities were verified additionally in strain PCY3 (Chevray and Nathans, 1992) that is used widely for determination of protein–protein interactions (G.Lametschwandtner, unpublished).

Mutations in the *ScPEX14* gene were not found in previous screens for mutants exhibiting *pex* phenotypes (Erdmann *et al.*, 1989; Van der Leij *et al.*, 1992; Elgersma *et al.*, 1993). Rather than identifying a novel *PEX* gene, we expected only subtle phenotypes (or none at all) in cells deleted at any gene obtained using our two-hybrid screen. That a *PEX* gene was identified subsequently in a screen for interaction partners underscores the urgent requirement for applying novel methods to elucidate peroxisomal protein translocation.

The integrity of the N-terminal myc-tagged Pex14p used for localization studies was verified by its ability to complement the phenotype of the corresponding deletion (Figure 5E). The appearance of Pex14p in the membrane pellet after carbonate extraction (Figure 7) lent further support for its suggested integration or tight association with the membrane. The lack of a predicted hydrophobic domain (Kyte and Doolittle, 1982) long enough to span membranes, as well as the high antigenicity of the tagged variant (Figure 5F) and its degradation by protease added to peroxisomes (Figure 8), indicate that the protein is not completely buried in the membrane, but is at least partially located at the surface. Post-translational modifications of the protein, the presence of short hydrophobic regions

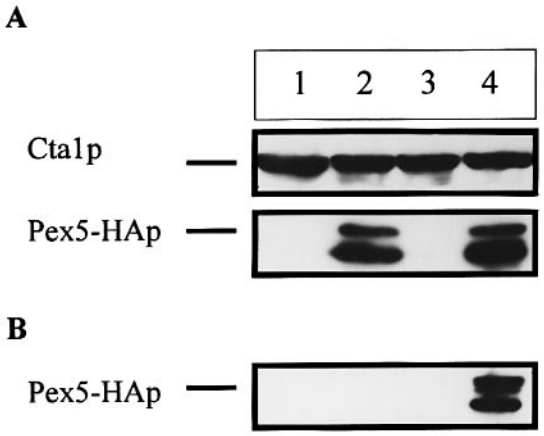

Fig. 9. Co-immunoprecipitation of Pex14p with Pex5p. Extracts were prepared from the oleate-cultured strain CB80 (1), as well as from oleate-cultured strains CB82 (Δ*pex5*), CB81 (Δ*pex14*) and CB83 (Δ*pex5*, Δ*pex14*) expressing either Pex5-HAp (2), myc-Pex14p (3) or both (4), respectively. Equal amounts of protein were subjected to Western blot analysis using antibodies against catalase A or the HA epitope (**A**). Equal amounts of extracts were immunoprecipitated using anti-myc antibodies, the immunocomplexes were isolated by protein G–Sepharose beads, separated by SDS–PAGE and the HA-tagged Pex5p was detected by immunoblotting using antibodies against the HA epitope (B).

(e.g. between amino acids 100 and 113, see Figure 1) and the possible interaction with membrane proteins may compensate for the lack of membrane-spanning domains.

The predicted coiled-coil domain in the middle of the protein may be responsible for oligomerization (Lupas, 1996), indicated by the two-hybrid interaction. Modifications at the N-terminus of Pex14p do not influence its function, though the N-terminal part may be essential for the interaction with Pex5p, since the three different *PEX14* inserts isolated in the screen were fused with the activation domain at the fourth codon. Close to the N-terminus the proline-rich motif PXXP (Feng *et al.*, 1994) is found, which could mediate the interaction with Pex13p via its SH3 domain. In this way, the N-terminal half of Pex14p may be a component of a multimeric complex consisting of at least Pex14p, Pex13p and Pex5p. This is in good agreement with the interaction of Pex13p with Pex5p (Elgersma *et al.*, 1996; Erdmann and Blobel, 1996; Gould

Table I. Two-hybrid interactions in strain HF7c demonstrated by growth on media lacking histidine (containing 20 mM 3-amino-1,2,4-triazol) or by development of blue color on filters soaked with X-Gal

Binding domain	Activation domain	Growth	β-Gal filter assay
pGBT9	pGAD424	–	n.d.
pGBT9	pGAD-*Sc*Pex14p	–	n.d.
pGBT9-*Sc*Pex14p	pGAD424	–	n.d.
pGBT9	pGAD-*Sc*Pex5p	–	n.d.
pGBT9-*Sc*Pex5p	pGAD424	+/–	white
pGBT9-*Sc*Pex5p	pGAD-*Sc*Pex14p	++	dark blue
pGBT9-*Sc*Pex14p	pGAD-*Sc*Pex5p	++	dark blue
pGBT9-*Sc*Pex7p	pGAD424	–	n.d.
pGBT9-*Sc*Pex7p	pGAD-*Sc*Pex14p	++	dark blue
pGBT9-*Sc*Pex13p	pGAD424	–	n.d.
pGBT9-*Sc*Pex13p	pGAD-*Sc*Pex14p	++	light blue
pGBT9-*Sc*Pex14p	pGAD-*Sc*Pex14p	++	light blue
pGBT9-Cox IV	pGAD-*Sc*Pex14p	–	n.d.
pGBT9-Cox IV-SKL	pGAD-*Sc*Pex14p	–	n.d.

++ indicates single colonies after 3 days at 30°C; +/– indicates single colonies after 5 days at 30°C; – indicates no growth; n.d. not determined.

et al., 1996), though, in contrast to Pex13p, for which no interaction with Pex7p has been reported (Erdmann and Blobel, 1996), Pex14p interacts with both receptors (Table I).

The function of this new peroxin is still unknown. The lack of peroxisomes in corresponding deletion mutants points to an essential role for Pex14p in the biogenesis of peroxisomes. Due to its membrane location and the many interactions detected, Pex14p most likely is a component of the translocation complex. As an interaction partner of both receptors, Pex14p may act as a cofactor for interactions of PTS receptors taking place at the membrane. There is still much controversy about the location of the two receptors involved in peroxisomal protein import (for review, see Subramani, 1996). Both proteins have been found associated predominantly with peroxisomes (Fransen *et al.*, 1995; Szilard *et al.*, 1995; Zhang and Lazarow, 1995), distributed between the cytosol and peroxisomes (Nuttley *et al.*, 1995; Van der Klei *et al.*, 1995) or located mainly in the cytosol (Marzioch *et al.*, 1994; Elgersma *et al.*, 1996; Gould *et al.*, 1996). However, recent evidence favors a model in which both Pex5p and Pex7p act as cycling receptors (Dodt and Gould, 1996; Rehling *et al.*, 1996) that bind only to the peroxisomal surface with a PTS-carrying substrate attached. The reaction for which Pex14p is essential could either be the docking event or the transfer of PTS-containing proteins from the receptors to the translocation machinery. Pex5p may dock to the organellar surface via Pex13p alone, via Pex14p alone or via a complex of both proteins, whereas Pex7p may use only Pex14p. Alternatively, upon binding to Pex14p a conformational change may be introduced to both cargo-loaded PTS receptors Pex5p and Pex7p that weakens the interaction with their substrates. This in turn may lead to the release and the transfer of peroxisomal matrix proteins to the translocation machinery and finally to their import into the peroxisomal matrix. Vacant receptors would cycle back to the cytosol and acquire a conformation competent for binding PTS-carrying proteins. The latter function as releasing factor would be unique for Pex14p among the few components of the peroxisomal import machinery identified so far. Both these hypothetical functions do not require that Pex14p interacts directly with substrate proteins, and such interactions have not been found yet (Table I).

The current model of post-translational import into preformed peroxisomes places Pex14p as a binding factor of substrate-loaded receptors to the organellar surface (either alone or together with Pex13p) or as a mediator of the transfer of targeted substrates to the import machinery at the peroxisomal surface. However, overexpression of the homologous protein in *H.polymorpha* results in aberrant vesicle formation and in the inability to grow on carbon sources requiring a functional peroxisomal compartment (Komori *et al.*, 1997). This vesicle formation may be taken as a first hint that the peroxisomal protein uptake mechanism makes use of vesicles or a vesicle fusion mechanism (McNew and Goodman, 1994; Subramani, 1996). It is interesting to speculate that Pex14p containing a coiled-coil domain may function like a SNARE, with the two ATPases Pex1p and Pex6p as possible NSF homologs (Wilson *et al.*, 1989; Erdmann *et al.*, 1991; Voorn-Brouwer *et al.*, 1993; Pfeffer, 1996, respectively).

As more components of the peroxisomal translocation machinery are discovered, a growing need for a description of their interaction is becoming apparent. Protein linkage maps, such as the ones presented here for Pex5p and Pex14p, will not only serve to explain the complex interactions among known components, but may also result in the identification of novel factors.

Materials and methods

Strains, media and culture conditions

The *Escherichia coli* strain HB101 (Bolivar and Backmann, 1979) was used for all transformations and plasmid isolations. The yeast strains used in this study were *S.cerevisiae* HF7c [*MAT**a**, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3/112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::*(*GAL4* 17mers)$_3$*-CYC1-lacZ*; Feilotter *et al.*, 1994], FY1679 (*MAT**a**/α, ura3-52/ura3-52, leu2-1/+, trp1-63/+, his3-200/+;* Winston *et al.*, 1995), CB80 (*MAT**a**, ura3-52, leu2-1, trp1-63, his3-200*), CB81 (*MAT**a**, ura3-52, leu2-1, trp1-63, his3-200, PEX14::Kan*MX4), CB82 (*MAT**a**, ura3-52, leu2-1, trp1-63, his3-200, PEX5::LEU2*) and CB83 (*MAT**a**, ura3-52, leu2-1, trp1-63, his3-200, PEX5::LEU2, PEX14::Kan*MX4). Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Inc., Detroit, MI), 2% glucose and amino acids (20–150 μg/ml) as required (SC-ura, SC-leu, SC-trp-leu, SC-trp-leu-his). For subcellular fractionation, electron and fluorescence microscopy, yeast cells were grown at 30°C with shaking in selective media with 0.5% glucose as sole carbon source until the glucose concentration was very low (~0.05%, usually 12–16 h), harvested by centrifugation and resuspended in twice the original volume of induction medium containing 30 mM potassium phosphate, pH 6.0, 0.3% yeast extract, 0.5% peptone and either 2% ethanol or 0.2% oleate (adjusted to pH 7.0 with NaOH) and 0.02% Tween-80. The plates contained 0.67% yeast nitrogen base without amino acids (Difco), 2% agar, amino acids as required and either 2% glucose, 2% ethanol, 2% glycerol or 2% potassium acetate, pH 6.0. Oleate plates contained 0.67% yeast nitrogen base without amino acids (Difco), 2% agar, 0.1% yeast extract, 0.5% potassium phosphate, pH 6.0, 0.125% oleate, 0.5% Tween-80 and amino acids as required. For phenotypic analysis, cells were grown overnight on 2% glucose medium, diluted and grown to mid-logarithmic phase in 0.5% glucose medium, washed twice with water and resuspended in 1/4 volume of water. Series of dilutions were made, and an aliquot was applied directly onto the surface of the plates. Plates were incubated at 30°C for 6 days.

Two-hybrid screen

Using the Matchmaker Two-Hybrid system protocol (Clontech, Palo Alto, CA), cells of yeast strain HF7c were transformed simultaneously

Table II. Oligonucleotides used in this study

Name	Oligonucleotide sequence	Notes
G-0260-S1	GAA AAC TCA AGT AAA ACA GAG AAG TTG TAA GGT GAA TAA GGA ATG CGT ACG CTG CAG GTC GAC	5′-side of *PEX14* and 5′-side of *Kan*MX4
G-0260-S2	CAA TTT CCG TTA AAA AAC TAA TTA CTT ACA TAG AAT TGC GCT AAT CGA TGA ATT CGA GCT CG	3′-side of *PEX14* and 3′-side of *Kan*MX4
G-0260-S3	CCA AAC AAC TGC AGC AGC TGG CTG	in *PEX14* promoter
G0260-S4	GCC GCT CAG CAC ATT TTA GTG C	in *PEX14* terminator
G-0260-S5	GGC AGC GTT GGT GGC ATC GC	in *PEX14* ORF
G-0260-S6	GGA ATC CGG TAA TGC GCA GGA C	in *PEX14* ORF
K2	GTC AAG ACT GTC AAG GAG GG	*Kan*MX4 module
K3	CAT CAT CTG CCC AGA TGC GAA G	*Kan*MX4 module
H198	CGT TAT CCA AAC TGA CGG ATC CTG ACA GGG TAC CCA TTT TCT TAA TTC TT	5′-side of *MLS1* ORF
H236	AAA CTC TAG AGG GAT CCC GAT CAT GGA CGT AGG AAG TTG C	5′-end of *PEX5* ORF
H237	ATA GAA GCT TAC TGC AGA AAT GCT AGC GCT GCC TCG CTC	3′-end of *PEX5* ORF
H278	ATT CGA TGA TGA AGA TAC CC	for sequencing 5′-side of the pGAD insert
H279	ACA GTT GAA GTG AAC TTG CG	for sequencing 3′-side of the pGAD insert
H306	GGA GGC TGC AGG CTC TTA TAA TTT GGA TAG TTC ATC C	3′-end of *GFP-SKL* ORF
H309	GAA TTC **ATG** GGA TCC AGT AAA GGA GAA GAA CTT TTC ACT GG	5′-end of *GFP* ORF
H344	GAT TTA AAG GAG AAT GCG GCC GCT GAT ATG GTT CT	3′-end of *PEX5* ORF
H345	AGA ACC ATA TCA GCG GCC GCA TTC TCC TTT AAA TC	H344 reverse strand
H346	ATA CCA GCA GGA TCC TGA CTA ACA	*PEX5* promoter
H347	AAG GTT GAA AGC ATG CTG ATA G	*PEX5* terminator
H348	GAA TAA GGA **ATG** AGC GGC CGC GTC AGT AAA GAT CG	5′-side of *PEX14* ORF
H349	CGA TCT TTA CTG ACG CGG CCG CTC ATT CCT TAT TC	H348 reverse strand
H352	CAG CTC TTG AAA CGG GAT CCC GTT ACT ATA	*PEX14* promoter
H353	GCC AAT ATA TTA GTC GAC TAA AAA TGC C	*PEX14* terminator
H355	GA TTC TAT ACT ATA AGG ATC CCT GCG **ATG** TC	5-side of *PEX13* ORF
H357	TGC GAA TAT ATG TCT GCA GAT ATT GAT GCA CTA	3′-side of *PEX13* ORF

Dotted lines indicate parts of the *Kan*MX4 module; solid lines indicate the created restriction sites; the nucleotides in bold indicate a start codon.

with plasmid pGBT9-*Sc*Pex5p (see below) and a genomic yeast library encoding fusion proteins between the Gal4 activation domain and random yeast genomic sequences (two-hybrid library, kindly provided by P.James, James *et al.*, 1996). Transformants were grown on selective media lacking tryptophan and leucine (SC-trp-leu). A total of 5×10^6 transformants harbored both plasmids (pGBT9-*Sc*Pex5p and a plasmid from the library). Among those, 216 candidates were able to grow on media without histidine in the presence of 20 mM AT (Sigma, Vienna, Austria) (SC-his-trp-leu with 20 mM AT). All the candidates were tested for activation of the integrated *lacZ* construct by β-galactosidase filter assay according to the Matchmaker protocol. From all colonies that turned blue (34 candidates), the library plasmid was isolated, retransformed into the strain HF7c and the growth tests were repeated as before but with 20, 35 or 55 mM AT. Among all isolated plasmids, seven allowed growth in the presence of 55 mM AT and gave rise to blue color using β-galactosidase assays.

Cloning procedures

Standard procedures were used for cloning and hybridization of DNA (Sambrook *et al.*, 1989). Linear fragments were isolated from the agarose gels as described (Dretzen *et al.*, 1981). Restriction enzymes were obtained from Boehringer-Mannheim (Vienna, Austria) and used as recommended. Double strand sequencing was performed using the T7 sequencing kit from Pharmacia (Uppsala, Sweden). Oligonucleotides were purchased from the Institute for Microbiology and Genetics, University of Vienna, Austria. For PCR, DynaZyme™ DNA polymerase from FINNZYMES OY (Espoo, Finland) was used. A list of oligonucleotides used in this study is given in Table II.

Disruption of *PEX14* in strain FY1679 was achieved using one-step gene disruption (Rothstein, 1983) with a fragment containing the *Kan*MX module from plasmid pFA6a-*Kan*MX4 (Wach *et al.*, 1994) and flanking sequences derived from the *PEX14* gene and provided within oligonucleotides G0260-S1 and G0260-S2. Verification of correct gene replacement was performed with genomic PCR from G418-resistant colonies with oligonucleotides G0260-S3, G0260-S4, G0260-S5, G0260-S6, K2 and K3. The protocol (Wach, 1996) is outlined in detail in the EUROFAN six-pack guidelines. The diploid was sporulated, tetrads analyzed and two haploid descendants (CB80, CB81) were used in this study.

The gene disruption construct for *PEX5* (pUC19-*pas10::LEU2*, kindly provided by H.Tabak) carrying a deletion of codons 123–420 was cut with *Pst*I and *Sac*I, the 3.1 kb fragment was isolated from a gel and subsequently transformed into strains CB80 and CB81 that gave rise to CB82 and CB83 respectively.

Plasmid pJR233 contains the GFP2 protein extended by the peroxisomal targeting signal SKL (kindly provided by T.Wenzel, San Diego, USA) under the control of the yeast *MLS1* promoter in the yeast–*E.coli* shuttle vector YEp352. Primers H306 and H309 and the template pGFP-PTS1 (Monosov *et al.*, 1996) were used in a PCR to produce a *Bam*HI–*Pst*I fragment encoding the GFP–SKL. This fragment was cloned into YEp352 (Hill *et al.*, 1986). Oligonucleotide-directed single-stranded DNA mutagenesis was used to create a *Kpn*I and a *Bam*HI site immediately after the start codon of *MLS1* (oligonucleotide H198) in an M13-derived phage containing a 2.3 kb *Sal*I fragment including the complete *MLS1* gene (Hartig *et al.*, 1992). The newly created fragment containing the *MLS1* promoter from –484 to +24 (*Eco*RI–*Bam*HI) was cloned in front of the GFP–SKL construct in YEp352, giving rise to plasmid pJR233.

Plasmid pGBT9-*Sc*Pex5p encodes the complete Pex5p fused in-frame to the Gal4-binding domain in the two-hybrid vector pGBT9 (Bartel *et al.*, 1993). Yeast genomic DNA was used as template in a PCR with oligonucleotides H236 and H237 to synthesize the 5′ half of *PEX5*; the resultant fragment of 1 kb was cloned into pBluescript® KS(–) (Promega, Madison, WI) using the newly created restriction sites for *Xba*I and *Hin*dIII. The DNA sequence was verified, and into the plasmid cut with *Hin*dIII and *Nhe*I the 3′ part of *PEX5* was cloned after isolation of a 1.1 kb *Hin*dIII–*Nhe*I fragment from pAH950 (Brocard *et al.*, 1994). The newly created 2.1 kb *Bam*HI–*Hin*dIII fragment harboring the complete

ORF of *PEX5* was isolated and cloned into pGBT9, resulting in plasmid pGBT9-*Sc*Pex5p.

Plasmid pGBT9-*Sc*Pex7p contains the 1.8 kb *Sma*I–*Pst*I fragment from pRS414-Gal4BD-*PEX7* (kindly provided by W.-H.Kunau, Bochum, Germany), and encodes the complete Pex7p fused in-frame to the Gal4-binding domain.

The *ScPEX13* ORF was obtained by PCR using the oligonucleotides H355 and H357. The PCR product was then cloned in the vector pGEM-T (pGEM-T SystemI, Promega). The plasmid pGBT9-*Sc*Pex13p contains the 1.2 kb *Bam*HI–*Pst*I fragment of the *PEX13* ORF fused in-frame to the Gal4-binding domain.

The *ScPEX14* ORF with its native promoter sequence was obtained by PCR using the oligonucleotides H348, H349, H352 and H353. The PCR fragments were cloned in the vector pGEM-T as before. A *Not*I site was generated in-frame with the start codon of *PEX14* by ligating the *Bam*HI–*Not*I promoter fragment (440 bp) and the *Not*I–*Sal*I 1163 bp fragment containing the *PEX14* ORF into the *Bam*HI–*Sal*I-cut YCplac33 vector (Gietz and Sugino, 1988), generating the vector YCp*PEX14*. Note that the third and fourth amino acids of Pex14p encoded by this plasmid are altered.

Plasmid pGAD-*Sc*Pex14p represents the original isolate from the two-hybrid library. Plasmid pGAD-*Sc*Pex5p was published previously (pAH950, Brocard *et al.*, 1994) and contains the ORF of *PEX5* between codons 78 and 612 fused in-frame to the Gal4 activation domain.

pGBT9-*Sc*Pex14p was derived from the plasmid pGAD-*Sc*Pex14p by isolating the *EcoRI* fragment (1.3 kb) and ligating it into pGAD424 (Clontech).

pGBT9-CoxIV and pGBT9-CoxIV-SKL were published previously (Brocard *et al.*, 1994). Both plasmids encode a protein fusion between the Gal4-binding domain and the subunit IV of cytochrome *c*-oxidase devoid of its mitochondrial targeting signal. The latter is extended by KNIESKL, the PTS1 from citrate synthase 2.

The plasmid YCp-myc-Pex14 was obtained by cloning a 375 bp *Not*I fragment containing nine copies of the myc epitope sequence (Zachariae *et al.*, 1996) into the vector YCp*PEX14* cut with *Not*I.

The *ScPEX5* ORF with its native promoter sequence was obtained by PCR using the oligonucleotides H344, H345, H346 and H347. The PCR fragments were cloned into the vector pGEM-T as before. A *Not*I site was generated in front of the stop codon of *PEX5* by ligating the *Bam*HI–*Not*I fragment (2160 bp) and the *Not*I–*Sph*I 125 bp fragment containing the *PEX5* ORF into the *Bam*HI–*Sph*I-cut YCplac22 vector (Gietz and Sugino, 1988), generating the vector YCp*PEX5*.

The plasmid YCp-Pex5-HA was obtained by cloning a 111 bp *NotI* fragment containing three copies of the HA epitope sequence (Zachariae *et al.*, 1996) into the vector YCp*PEX5* cut with *NotI*.

Immunoprecipitation

Extracts for immunoprecipitation were prepared as described (Lamb *et al.*, 1994). Cells were cultured in oleate-containing medium (2×10^7 cells/ml). Extracts (9 mg proteins in 0.5 ml) were incubated for 2 h on ice with the 9E10 hybridoma supernatant (100 µl). Protein G–Sepharose (Pharmacia) washed in incubation buffer was added (200 µl of 50% slurry) and the whole mixture was incubated for 1 h at 4°C on a rolling wheel. Protein G–Sepharose beads were separated by low speed centrifugation and washed five times in incubation buffer. The proteins bound were separated by SDS–PAGE and analyzed by standard immunoblotting techniques (Bollag and Edelstein, 1991).

Other methods

Total yeast RNA was prepared as described (Richter *et al.*, 1980) and subjected to Northern analysis by standard procedures (Sambrook *et al.*, 1989).

Preparation of the organellar pellet and immunoblot analysis were done as described (Kragler *et al.*, 1993). Peroxisomes were purified from the organellar pellet by sucrose gradient centrifugation (Hartig *et al.*, 1990), concentrated through a sucrose cushion and extracted either with low salt (10 mM Tris–HCl, pH 8.0), high salt (0.5 M KCl in 10 mM Tris–HCl, pH 8.0) or 0.1 M sodium carbonate (pH 11.5) as described (Erdmann and Blobel, 1995). Equivalent amounts of each fraction were precipitated by 10% trichloroacetic acid (final concentration), separated by SDS–PAGE and analyzed by standard immunoblotting techniques (Bollag and Edelstein, 1991). Total protein was measured using the protein assay from Bio-Rad (Bio-Rad, Munich, Germany).

Protease protection was performed on the organellar pellet from oleate-grown cells as described (Crane *et al.*, 1994).

Polyclonal antibodies against GFP were purchased from Clontech, antibodies against catalase A were from rabbit, and rabbit antibodies against thiolase (Erdmann and Kunau, 1994) were a kind gift from Dr W.-H.Kunau, Bochum, Germany. The antibody against the SH3 domain of Pex13p was kindly provided by Dr R.Erdmann (Erdmann and Blobel, 1996) and the hybridoma supernatants from 9E10 (anti-myc) and 12CA5 (anti-HA) were kindly supplied by Dr A.Gartner, Vienna. For detection of the respective proteins on Western blots, the primary antibodies were diluted (anti-catalase 1:7000, anti-thiolase 1:7000, anti-Pex13p 1:10 000, myc hybridoma supernatant 1:50, HA hybridoma supernatant 1:50) in 4% w/v non-fat dry milk in Tris-buffered saline. The secondary antibodies, either anti-mouse or anti-rabbit horseradish peroxidase-conjugated Ig (from donkey; Amersham), were diluted 1:7000 and visualized according to the manufacturer's instructions (ECL kit, SuperSignal™ Substrate Western Blotting, Pierce, Rockford, IL).

Electron microscopy and immunoelectron microscopy was done as described (Kragler *et al.*, 1993) but with a lower concentration of glutaraldehyde (0.2% final concentration). Sections were viewed in a Zeiss electron microscope EM 900 (Zeiss, Oberkochen, Germany). For control of the specificity of the labeling procedures for immunoelectron microscopy, the respective primary antibody in the incubation mixture was replaced by pre-immune serum.

Immunofluorescence was performed as described (Pringle *et al.*, 1991), with antibodies against thiolase (diluted 1:200) and fluorescein isothiocyanate-conjugated antibodies against rabbit IgG (Sigma, diluted 1:50). Fluorescence of cells was viewed with a Leitz Aristoplan microscope (Leica, Wetzlar, Germany) and photographed.

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