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Research Article

Pichia pastoris Pex14p, a phosphorylated peroxisomal membrane protein, is part of a PTS–receptor docking complex and interacts with many peroxins

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Abstract

The peroxisomal protein import machinery plays a central role in the assembly of this organelle in all eukaryotes. Genes encoding components of this machinery, termed peroxins or Pex proteins, have been isolated and characterized in several yeast species and in mammals, including humans. Here we report on one of these components, Pex14p, from the methylotrophic yeast *Pichia pastoris*. Work in other organisms has shown that Pex14p is located on the cytoplasmic surface of the peroxisomal membrane and binds peroxisomal targeting signal (PTS) receptors carrying proteins bound for the peroxisomal matrix, results that have led to the hypothesis that Pex14p is a receptor-docking protein. *P. pastoris* Pex14p (*Pp*Pex14p) behaves like an integral membrane protein, with its C-terminus exposed on the cytosolic side of the peroxisomal membrane. *Pp*Pex14p complexes with many peroxins, including Pex3p (Snyder *et al.*, 1999b), Pex5p, Pex7p, Pex13p, Pex17p, itself, and a previously unreported peroxin, Pex8p. A portion of Pex14p is phosphorylated, but both phosphorylated and unphosphorylated forms of Pex14p interact with several peroxins. The interactions between Pex14p and other peroxins provide clues regarding the function of Pex14p in peroxisomal protein import. Copyright © 2001 John Wiley & Sons, Ltd.

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Introduction

Studies of the mechanism of peroxisomal protein import and biogenesis using a variety of different model systems have led to the discovery of 23 peroxins encoded by peroxisome biogenesis genes (*PEX* genes). Of these, at least 13 are conserved in humans and 11 are implicated in fatal human peroxisome biogenesis disorders, underscoring the importance of this organelle (Waterham and Cregg, 1997; Subramani, 1998; Wanders, 1999; Brown *et al.*, 2000).

All peroxisomal proteins are nuclear-encoded, synthesized on free polysomes and post-translationally imported into the organelle via one of several peroxisomal targeting signals (PTSs) (Waterham and Cregg, 1997). The first and most widely used signal, PTS1, is composed of a tripeptide sequence (SKL and conservative variants) present at the extreme carboxy-terminus of many peroxisomal matrix proteins (Subramani, 1998; Geraghty *et al.*, 1999). PTS2, a second matrix targeting signal, is less common and is located on

a nonapeptide (R/K, L/V/I, X_5, H/Q, L/A) near the NH_2-terminus (Subramani, 1998). Both PTS1 and PTS2 have been evolutionarily conserved from yeast to humans. Peroxisomal membrane protein targeting signals (mPTSs) are less well understood. However, it is clear that they are targeted to the peroxisome by a mechanism that is different from that of matrix proteins.

The PTS1 and PTS2 sequences on cargo proteins destined for the peroxisomal matrix are recognized in the cytosol by specific receptors, Pex5p and Pex7p (Subramani, 1998). Recently, two putative PTS1- and PTS2-receptor docking proteins were identified. These peroxins are located on the peroxisomal membrane surface and bind Pex5p and Pex7p. Pex13p, an integral membrane protein, binds Pex5p through its cytosolic Src homology 3 (SH3) domain and Pex7p through a portion of its amino-terminus that does not contain the SH3 domain (Elgersma *et al.*, 1996; Erdmann and Blobel, 1996; Gould *et al.*, 1996; Girzalsky *et al.*, 1999). The second potential docking protein is Pex14p. In *Saccharomyces cerevisiae*, Pex14p interacts with Pex5p, Pex7p, Pex13p, Pex17p, and itself (homo-oligomerization) (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Huhse *et al.*, 1998; Girzalsky *et al.*, 1999). Human Pex14p interacts with Pex5p and possibly also Pex13p (Fransen *et al.*, 1998; Will *et al.*, 1999), but Pex7p was not tested and no mammalian homologue of Pex17p has been described yet. Pex14p in *S. cerevisiae* was reported to behave as either a peripherally associated peroxisomal membrane protein (Albertini *et al.*, 1997; Girzalsky *et al.*, 1999) or an integral membrane protein (Brocard *et al.*, 1997). In *Hansenula polymorpha*, human and Chinese hamster ovary (CHO) cells, Pex14p behaved as an integral membrane protein (Komori *et al.*, 1997; Fransen *et al.*, 1998; Shimizu *et al.*, 1999; Will *et al.*, 1999). The series of events that occur between the docking of cargo-bound PTS receptors with the peroxisome-associated docking proteins and the translocation of the cargo into the peroxisome matrix remains to be elucidated.

Here we report the cloning of the *Pichia pastoris PEX14* gene and the characterization of its product, Pex14p. We define the subcellular location of Pex14p and its requirement for the import of both PTS1 and PTS2 proteins but not for the targeting of peroxisomal integral membrane proteins. Pex14p is found complexed with Pex3p (Snyder *et al.*, 1999b), Pex5p, Pex7p, Pex8p, Pex13p, Pex17p, and with itself. We also report that a portion of Pex14p is phosphorylated.

Materials and methods

Strains, media and microbial techniques

P. pastoris strains used in this study are listed in Table 1. Media and conditions for culturing were as described previously (Johnson *et al.*, 1999). The sporulation and mating procedures for classical genetic manipulation of *P. pastoris* have been described previously (Cregg *et al.*, 1998). Transformation of *P. pastoris* was done by electroporation according to Cregg and Russell (1998). Cultivation of *Escherichia coli* and standard recombinant DNA techniques were performed essentially as described previously (Sambrook *et al.*, 1989).

Cloning and sequence analysis of *PEX14*

To isolate the *PEX14* gene, the *P. pastoris pex14-1 his4* mutant JC400 was transformed with a *P. pastoris* genomic DNA library (Liu *et al.*, 1995). Two of the recovered plasmids, pMJPEX14-6 and pMJPEX14-11, restored histidine prototrophy and methanol growth upon transformation back into JC400. Restriction analysis showed the inserts in pMJPEX14-6 and in pMJPEX14-11 to be approximately 6.3 and 9.2 kb, respectively. DNA sequencing of both strands of the insert in pMJPEX14-6 was performed at the Oregon Regional Primate Research Center (Beaverton, OR). Open reading frames (ORFs) were identified using MacVector software. The BLAST-BEAUTY Network Service of the National Center for Biotechnology Information was used to search for sequence similarities in protein databases. Sequence alignments were done using MacVector ClustalW. ORF1 was subcloned under the control of the *P. pastoris PEX8* promoter into pK312 (Johnson *et al.*, 1999) and transformed into JC400 to test for the ability of this ORF to complement methanol and oleate growth defects. COILS (Lupas *et al.*, 1991) was used to search for coiled-coil motifs.

Construction of a *PEX14* deletion strain

To delete the wild-type *PEX14* gene, the *S. cerevisiae ARG4* gene (*SARG4*) (Beacham *et al.*, 1984) was amplified by polymerase chain reaction (PCR) with

Table 1. Strains used in this study

Name	Genotype	Comments	Source (if other than this study)
JC140	*pex14-1 arg4*		Johnson *et al.*, 1999
JC227	*ade1 arg4*		
JC400	*pex14-1 his4*		
JC401	*pex14-1 his4* (pMJKORF1) P_{PEX8}*PEX14*	Rescued	
JC403	*pex14*Δ::*SARG4 arg4*	Deletion	
JC404	*pex14*Δ::*SARG4 arg4 his4*	Deletion	
JC405	*pex14*Δ::*SARG4 arg4 ade4*	Deletion	
JC406	*pex14*Δ::*SARG4 arg4 his4* (pMJKORF1)	*PEX14* rescued	
JC407	*pex14*Δ::*SARG4 arg4 his4* (pMJPHORF1)	*PEX14* rescued	
JC408	*pex14-1 his4* (pHW017)	P_{AOX}Luc	
JC409	*pex14*Δ::*SARG4 arg4 his4* (pHW017)	P_{AOX}Luc	
JC410	*pex14-1 his4* (pMJJSORF1)(pHW017)	P_{AOX}Luc *PEX14* rescued	
JC411	*pex1 his4* (pHW017)	P_{AOX}Luc	
JC412	*his4* (pHW017)	P_{AOX}Luc	
JC413	*pex14-1 his4* (pOPGP1)	P_{PEX8}EGFP–PTS1	
JC414	*pex14*Δ::*SARG4 arg4 his4* (pOPGP)	P_{PEX8}EGFP–PTS1	
JC415	*pex14-1 his4* (pMJJSORF1)(pOPGP)	P_{PEX8}EGFP–PTS1 *PEX14* rescued	
JC416	*pex1 his4* (pOPGP)	P_{PEX8}EGFP–PTS1	
JC417	*his4* (pOPGP)	P_{PEX8}EGFP–PTS1	
JC418	*pex14-1 his4* (pTW65)	P_{ACO}PTS2–EGFP	
JC419	*pex14*Δ::*SARG4 arg4 his4* (pTW65)	P_{ACO}PTS2–EGFP	
JC420	*pex14-1 his4* (pMJJSORF1) (pTW65)	P_{ACO}PTS2–EGFP rescued	
JC421	*pex14-1 his4* (pLC303)	P_{AOX}mPTS–EGFP	
JC422	*pex14*Δ::*SARG4 arg4 his4* (pLC303)	P_{AOX}mPTS–EGFP	
JC423	*pex14-1 his4* (pMJKORF1) (pLC303)	P_{AOX}mPTS–EGFP *PEX14* rescued	
JC424	*pex1 his4* (pLC303)	P_{AOX}mPTS–EGFP	
JC425	*his4* (pLC303)	P_{AOX}mPTS–EGFP	
JC426	*ade1/ADE1 arg4/arg4 his4/HIS4*	Diploid strain	

primers that contained 75 bp of *PEX14* 5′ flanking region along with 20 bp of *SARG4* 5′ sequence and 77 bp of *PEX14* 3′ flanking region along with 20 bp of *SARG4* 3′ sequence. The 5′ forward primer sequence was 5′-ATCATATTTAAGGCC CATCTTCCCCAACCTCGAGTAGTGTTTGTTG TTGTG-CCATCTGTTATCAAGCCGTCCGAAA ATAAATGGTTGGCGCAGGC-3′ and consisted of 75 bases immediately upstream of the *PEX14* methionine initiator codon ATG (−75 to −1) followed by 20 bases (−375 to −352) upstream of the *SARG4* ATG. The 3′ reverse primer sequence was 5′-ATTTCAATGCATAATGCGCCCAGAAG CTGAGCTTCTCAAGTAAGT-AACTTTCTATTA CCGTTGATCAACAGCGATACATACGACTTT GGGAGGTTACAA-3′ and consisted of 77 bases downstream of *PEX14* (+26 to +102 from the translational stop codon) followed by 20 bases of *SARG4* 3′ flanking region (+327 to +346 from the translational stop codon). The template used was pYM25, which is composed of a 3.1 kb *Hin*dIII fragment encoding the *SARG4* gene inserted into the *Hin*dIII site of pBR322 (Cregg *et al.*, 1989). PCR yielded an expected 2256 bp product (2104 bp of *SARG4* plus 75 bp of *PEX14* 5′ flanking region plus 77 bp of *PEX14* 3′ flanking region). This PCR product was transformed into the *P. pastoris arg4/arg4* diploid strain JC426. Arginine diploid prototrophs were sporulated, and spores germinated on YND (Yeast Nitrogen Base dextrose) plates, supplemented with adenine and histidine, and spore products were screened for ability to grow on YNM (Yeast Nitrogen Base methanol) plates. Mut$^-$ strains were examined for correctly targeted genomic integration of *SARG4* and deletion of *PEX14* by PCR. For PCR analysis, total genomic DNA was recovered from three putative *pex14*Δ*::SARG4* strains. PCR was performed using primer A, a forward primer (5′-CCCCAACCTC GAGTAGTG-3′) composed of nucleotides −54 to −37 of the *PEX14* 5′ flanking region, in combination with either reverse primer B (5′-AC

GTATTCTTTATGCTCTCA-3′), which is the complement of nucleotides 614–633 within the *PEX14* ORF, or in combination with reverse primer C (5′-TGTATGAAACCAAATTCT-3′), which is the complement of nucleotides 1180–1196 of *SARG4*. PCR with primers A and B on wild-type genomic DNA as a template was predicted to yield a product composed of *PEX14* −54 to –633 (687 bp). In contrast, using primers A and B with *pex14Δ::SARG4* strain genomic DNA as a template should yield no PCR product. PCR with primers A and C and wild-type genomic DNA as a template should yield no PCR product, while primers A and C with *pex14Δ::SARG4* strain genomic DNA as a template should yield a PCR product of 1250 bp (54 bp of *PEX14* 5′ flanking plus 1196 bp of *SARG4*). All three putative *pex14Δ::SARG4* strains generated the predicted PCR fragment products (data not shown).

Preparation of anti-Pex14p antibodies

The carboxy-terminal 146 amino acids of Pex14p were expressed in *E. coli* as a fusion with maltose binding protein (MBP) using the Protein Fusion and Purification System supplied by New England Biolabs (Beverly, MA). To subclone this 446 bp fragment, which included the *PEX14* translational stop codon and five additional 3′ bp, PCR was performed using primers that added a 5′ *Bam*HI site (5′-CGCGGATCCTCTGTACCAATAAGGACAC AACTC) and a 3′ *Pst*I site (5′-TTAACTGCAG GACAACTCAGCTTTGAGCTGCCAACTG) to the *PEX14* fragment. The PCR product was inserted into *Bam*HI- and *Pst*I-cut pMAL-c2, resulting in pMJMEND-6. Purified MBP–Pex14p fusion protein was then used to immunize rabbits (Josman Laboratories, Napa, CA).

Plasmid constructions

All plasmids used for this work are listed in Table 2. pMJKORF1, a vector capable of expressing the *PEX14* ORF in *P. pastoris* under the control of the *P. pastoris PEX8* promoter (P_{PEX8}) (Liu *et al.*, 1995), was constructed as follows: *Mfe*I sites were added to both ends of the *PEX14* ORF by PCR, using as forward primer 5′-GGCGGCCAATTG ATGTCCAGTATACGTGAAGAAATG-3′ and as reverse primer 5′-CGATACCAATTGTCAGCT TTGAGCTGCCAACTGCCAAG-3′. The PCR product was inserted at the *Eco*RI site of pK312 (Johnson *et al.*, 1999). The vector was linearized within *HIS4* by digestion with *Sal*I prior to transformation into *P. pastoris*.

pMJJSORF1, a second vector capable of expressing the *PEX14* ORF under the control of P_{PEX8}, was constructed by inserting the *PEX14* PCR product described above at the *Eco*RI site of pJS1. pJS1 is the zeocine-resistance selection vector pPICZ B (Invitrogen, San Diego, CA) with a *Bgl*II–*Eco*RI fragment containing P_{AOX} excised and replaced with a *Bam*HI–*Eco*RI fragment from pK312 carrying P_{PEX8}. The vector was linearized within *PEX14* by digestion with *Bgl*II prior to transformation into *P. pastoris*.

pMJSORF7C, a vector capable of expressing the *PEX14* ORF in *E. coli*, was constructed as follows: a PCR product composed of the *PEX14* ORF with an added 5′ *Nco*I site and a 3′ *Eco*RI site was made by PCR using the 5′ forward primer 5′-TAGCGTC CATGGCCAGTATACGTGAAGAAATG-3′ and the 3′ reverse primer 5′-CGATACCAATTGT CAGCTTTGAGCTGCCAACTGCCAAG-3′. The resulting PCR product was inserted into *Nco*I- and *Eco*RI-digested pSE380 (Invitrogen).

Two-hybrid clones containing *PEX17* and subdomains are described elsewhere (Snyder *et al.*, 1999b). A full-length clone of *PEX14* was amplified by PCR [primers 2h14u (GCGGATCCAT GTCCAGTATACGTGAAGAAATG) and 2h14d (GATCCTGCAGGCTTTGAGCTGCCAACTGCC)] and inserted as a *Bam*HI–*Pst*I fragment into pKNSD55 (two-hybrid binding domain vector) and pKNSD52 (two-hybrid activating domain vector) cut with *Bam*HI and *Pst*I, creating p2HBD14 and p2HAD14. Full-length *PEX13* and the SH3(247B380) domain were amplified by PCR and introduced into pKNSD55 and pKNSD52 as follows: *PEX13* [primers 2h13u (GTCCAGATC TATGAGACTCATCAGCTCC) and 2h13d (CG CGACTACTTTATGTCTTCATCTTCT)] was cut with *Spe*I and *Bgl*II and inserted into vectors cut with *Spe*I and *Bam*HI, creating p2HBD13 and p2HAD13; *PEX13*(SH3) [primers P13sh3u (GTCCAGATCTAAGAAATTAATTGCTCATCT TGC) and 2h13d] was cut with *Spe*I and *Bgl*II and cloned into vectors cut with *Spe*I and *Bam*HI, creating pBD13sh3 and pAD13sh3.

The strain expressing Pex17-HAp is described elsewhere (Snyder *et al.*, 1999b). The HA epitope was cut from the *PEX17–HA* plasmid (Snyder *et al.*, 1999b) by cutting with *Xma*I and *Pst*I and was

Table 2. Plasmids used in this study

Name	Comment	Source (if other than this study)
P. pastoris–E. coli shuttle vectors		
pMJPEX14-6	pYM8 + *Pp* library fragment (6335 bp)	
pMJPEX14-11	pYM8 + *Pp* library fragment (9.2 kb)	
pMJPHORF1	PPHIL–A1 + *PEX14* (P_{AOX})	
pMJKORF1	pK312 + *PEX14* (P_{PEX8})	
pMJJSORF1	pJS1 (pPICZ-B but P_{PEX8}) + *PEX14*	
pMJMEND-6	pMALc-2 + #914–1360 of *PEX14*	
pHW017	pHIL-A1 Luc	Waterham *et al.*, 1996
pOPGP	pK312 with EGFP–AKL (P_{PEX8})	Johnson *et al.*, 1999
pLC303	pPICZ-B with mPTS–EGFP (P_{AOX})	Johnson *et al.*, 1999
pTW65	pHIL-D2 with PTS2–EGFP (P_{ACO})	Johnson *et al.*, 1999
pK312	pHIL-A1 but with P_{PEX8} in place of P_{AOX}	Johnson *et al.*, 1999
p17HA	*PEX17* HA tagged	
p13HA	*PEX13* HA tagged	
pHA7	*PEX7* HA tagged	
S. cerevisiae two-hybrid system vectors		
p2HBD14	Binding domain (BD) containing full-length (FL) *PEX14*	
p2HAD14	Activating domain (AD) containing FL PEX14	
p2HB13	BD containing FL *PEX13*	
p2HAD13	AD containg FL *PEX13*	
pBD13sh3	BD containing the SH3 domain of *PEX13*	
pAD13sh3	AD containing the SH3 domain of *PEX13*	
p2H17	BD containing FL *PEX17*	Snyder *et al.*, 1999b
p2H17NB	BD containing *PEX17* [1–124]	Snyder *et al.*, 1999b
p2H17lum	BD containing *PEX17* [1–59]	Snyder *et al.*, 1999b
p2H17cyt	BD containing *PEX17* [52–267]	Snyder *et al.*, 1999b
E. coli expression vector		
pMJSORF7C	pSE380 + *PEX14* (*E. coli* expression)	

cloned into pIB1 (a gift of Ben Glick, University of Chicago) cut with the same, creating pIBHA. *PEX13* was amplified by PCR with primers TAG13u (GCGCCAATTGACACTTTCACCCCGCGTTTG) and TAG13dn (GCGCCCCGGGTGTCTTCATC TTCTGAAATTCTG), cut with *Mfe*I and *Xma*I, and cloned into pIBHA cut with *Eco*RI and *Xma*I, creating p13HA. This plasmid was linearized by cutting with *Sal*I and integrated into the *HIS4* locus of the *pex13*Δ*::ZEO* strain (laboratory collection), creating strain SWS13HA. Pex13–HAp complements the *pex13*Δ strain for growth on methanol and oleate. *HA–PEX7* was created by two-step PCR. Primers TAG7u (GCGCAGATCTTACAT GCCCGGGCGCATCTTTTAC) and HA7d (CG TTTGTTTGGAACTTAAACATGCGGCCGCAC TGAGCAG) were used to amplify the HA tag, and primers TAG7d (GCGCCAATTGTTAC TGTTGTCTCTGTGTATTC) and HA7u (CTGC TCAGTGCGGCCGCATGTTTAAGTTCCAAAC AAACG) were used to amplify *PEX7*. The two products were gel-purified and combined as a template for PCR with primers TAG7u and TAG7d, creating the full-length *HA–PEX7*. This was cut with *Bam*HI and *Eco*RI and cloned into p21.43 (Snyder *et al.*, 1999a) cut with *Bgl*II and *Eco*RI, creating pHA7. pHA7 was linearized with *Sal*I and integrated into the *HIS4* locus of the *pex7*Δ strain (Elgersma *et al.*, 1998), creating SWS7HA, which was complemented for growth on oleate.

Biochemical methods

Peroxisomal alcohol oxidase (AOX), catalase (CAT), and mitochondrial cytochrome *c* oxidase were assayed for activity or protein according to previously described methods (Johnson *et al.*, 1999). Luciferase activity was determined using the TB101 Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's specifications. Immunoblotting experiments were performed

with specific polyclonal (unless otherwise stated) antibodies against *P. pastoris* Pex14p, Pex5p, Pex8p, AOX, CAT or thiolase (a gift from W.H. Kunau, Ruhr University, Bochum, Germany), or monoclonal antibodies against HA. Primary antibodies and dilutions used were as follows: α-Pex5 (1 : 5000), α-Pex8p (1 : 10,000), α-Pex14p (1 : 10,000), rat-α-HA (1 : 1000). The primary antibodies were detected using either protein A conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA), or goat anti-rat antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA), or goat anti-rabbit antibody conjugated to horseradish peroxidase and preabsorbed against rat sera (Jackson ImmunoResearch Laboratories). Detection was done using either an ECL (Amersham Corporation, Arlington Heights, IL) or Western Light kit (Tropix, Bedford, MA) according to the manufacturer's protocols. Cell lysates and subcellular fractionation were performed as previously described (Johnson *et al.*, 1999). The sucrose density gradient was prepared according to a previously published procedure (Waterham *et al.*, 1996). Fluorescence microscopy was performed as previously described (Johnson *et al.*, 1999); potato acid phosphatase (PAP) treatments were done as previously described (Elgersma *et al.*, 1997); Yersinia protein tyrosine phosphatase (YOP) treatments were performed according to the manufacturer's instructions (NEB, Beverly, MA).

Membrane extraction and protease protection

For carbonate and detergent extractions of membrane preparations, 1.5 ml samples containing 0.8–1.0 mg of oleate-induced $30\,000 \times g$ pellet preparation were adjusted to a final concentration of 0.1 M Na_2CO_3, pH 11.5, or 1% Triton X-100 in a buffer of 0.01 M Tris–HCl, pH 8.0, 1 mM PMSF, 1 mM leupeptin, and 1 mM aprotinin. Samples were incubated for 30 min on ice and centrifuged in a SW60.1 Ti rotor at $100\,000 \times g$ for 60 min. Resulting pellet and supernatant fractions were precipitated with 10% trichloroacetic acid and washed twice with acetone. Equal volumes of supernatant and pellet fractions were loaded in all lanes for SDS–PAGE. Proteins were detected by immunoblotting.

Protease protection assay samples were created previously (Snyder *et al.*, 1999b).

Immunoprecipitation

Immunoprecipitation and crosslinking with DSP [dithiobis(succinimidyl propionate)] (Pierce, Rockford, IL) were performed from 5 A_{600} units of oleate-grown cells as described previously (Rieder and Emr, 1997). The lysis buffer contained the following protease and phosphatase inhibitors (final concentration or dilution factor used): 50 nM okadaick acid, 2.5 mM sodium azide, 2.5 mM sodium fluoride, 12.5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 : 400 of protease and phosphatase inhibitor cocktail for fungal and yeast extracts (Sigma, P8215, St. Louis, MO). For immunoprecipitations, 10 μl affinity-purified anti-Pex14p antisera (purified according to Harlow and Lane, 1988) and 5 μl anti-HA (Covance, Richmond, CA) was used per immunoprecipitation.

To visualize Pex14p on an immunoblot following immunoprecipitation with either Pex13–HAp or Pex17–HAp or HA–Pex7p, the secondary antibody used was a goat anti-rabbit antibody conjugated to horseradish peroxidase and pre-absorbed against multiple sera (Jackson ImmunoResearch Laboratories, #111-035-144, West Grove, PA).

Miscellaneous methods

Cloning vectors, tester strains and screening by two-hybrid analysis were performed as described previously (Faber *et al.*, 1998). Electron microscopy was performed as previously described (Waterham *et al.*, 1996).

Results

Cloning and identification of the *P. pastoris PEX14* gene

The *PEX14* gene was cloned by functional complementation of the *P. pastoris per14-1 his4* mutant (JC400) (Johnson *et al.*, 1999) using a *P. pastoris* genomic DNA library (Liu *et al.*, 1995). Two plasmids, pMJPEX14-6 and pMJPEX14-11, were recovered that contained common *P. pastoris* restriction fragments and transformed the *pex14-1 his4* strain simultaneously to His^+ and proficiency for methanol utilization (Mut^+), indicating that both plasmids most likely contained the *PEX14* gene. DNA sequencing of the 6.3 kb DNA insert in pMJPEX14-6 revealed three long ORFs. ORF2 potentially encoded a polypeptide with 65% identity and 78% similarity to the *S. cerevisiae*

homoaconitase. The putative ORF3 product showed no sequence similarity to any other protein in the databases. The putative ORF1 product was identified as a possible orthologue of the *H. polymorpha* and *S. cerevisiae PEX14* products (Albertini *et al*., 1997; Brocard *et al*., 1997; Komori *et al*., 1997). ORF1 was subcloned under the control of a *P. pastoris PEX8* promoter, and the resulting vector, pMJKORF1, complemented the *pex14-1 his4* strain, indicating that ORF1 was most likely *PEX14*.

ORF1 was predicted to encode a polypeptide of 425 amino acids with a calculated molecular mass of 47 kDa. Alignment of the deduced amino acid sequence of ORF1 to known Pex14 proteins is shown in Figure 1, and the degree of similarity is considered below (see Discussion). The yeast Pex14ps all share a conserved class II SH3-ligand binding motif (xPPLPxR, Figure 1, indicated by overline), which has been shown in other proteins, and in *S. cerevisiae* Pex14p (*Sc*Pex14p) (Girzalsky *et al*., 1999), to facilitate interaction with SH3 domains (Feng *et al*., 1994). Interestingly, Pex14p from human, CHO and rat cells do not have this motif (Fransen *et al*., 1998; Shimizu *et al*., 1999; Will *et al*., 1999). Programmes designed to predict transmembrane segments gave mixed results. For example, three prediction programmes found no domain capable of spanning the membrane (Hirokawa *et al*., 1998; Sonnhammer *et al*., 1998; Tusnady and Simon, 1998). In contrast, two other programmes (Hoffman and Stoffel, 1993; von Heijne, 1992) found a region, amino acids (aa) 100–124, with a potential to span the membrane once, despite the presence of charged residues within this region. Therefore the membrane-spanning capability of the PpPex14p is unclear from sequence analysis (see Discussion).

To examine the phenotype of a *PEX14* null mutant, a deletion strain was constructed. A DNA fragment, in which all of ORF1 was replaced by a fragment containing the *S. cerevisiae ARG4* gene (*SARG4*), was first constructed using the super-primer PCR method and then used to replace ORF1 in the *P. pastoris* genome, as described in Materials and methods (Shoemaker *et al*., 1996). One of the resulting strains, JC405, was shown genetically and by PCR analysis to contain the predicted *pex14*Δ*::SARG4* allele and was used in all further studies.

P. pastoris pex14 mutants lack normal peroxisomes but contain peroxisome remnants

To investigate the function of Pex14p, the phenotypical and morphological characteristics of the *pex14-1* and *pex14*Δ strains were examined and compared to wild-type *P. pastoris*. Both of the *pex14* mutants grew at a similar rate to wild-type cells on glucose, glycerol or ethanol, but were specifically unable to grow on methanol or oleate (data not shown). Electron micrographs of methanol- and oleate-induced cells of the *pex14-1* and *pex14*Δ strains revealed the absence of normal peroxisomes (Figure 2C, D, E, F). Instead, small vesicular structures were induced that appeared similar to peroxisomal remnants or ghosts observed in other *P. pastoris pex* mutants. That these vesicular structures are most likely peroxisomal remnants was supported by fluorescence microscopy studies of *pex14* mutants expressing a known peroxisomal integral membrane protein (Pex2p) (Waterham *et al*., 1996) fused to green fluorescent protein (EGFP), which showed a punctate pattern typical of remnant structures in *P. pastoris pex* mutants (Johnson *et al*., 1999) (Figure 3F, I). All growth and morphology defects disappeared in both methanol- and oleate-grown cells of a *pex14-1* strain transformed with a vector expressing *PEX14* under control of the *PEX8* promoter (*PEX14* rescued strain) (cf. Figure 2G, H, with Figure 2A, B; cf. Figure 3J, K, L with Figure 3A, B, C).

Pex14p is required for import of PTS1 and PTS2 proteins, but not for the targeting of peroxisomal membrane proteins

The function of specific PTS pathways in the *pex14-1* and *pex14*Δ mutants was investigated through a combination of subcellular fractionation experiments and EGFP-based fluorescence microscopy studies. For subcellular fractionation, cells were induced in oleate, spheroplasted, homogenized and centrifuged at $30\,000 \times g$. The resulting organellar (primarily peroxisomal and mitochondrial) pellet and cytosolic supernatant fractions were then assayed for selected peroxisomal proteins. Cytochrome *c* oxidase, a mitochondrial marker protein, was used as a control to confirm the general integrity of the organelles in the pellet.

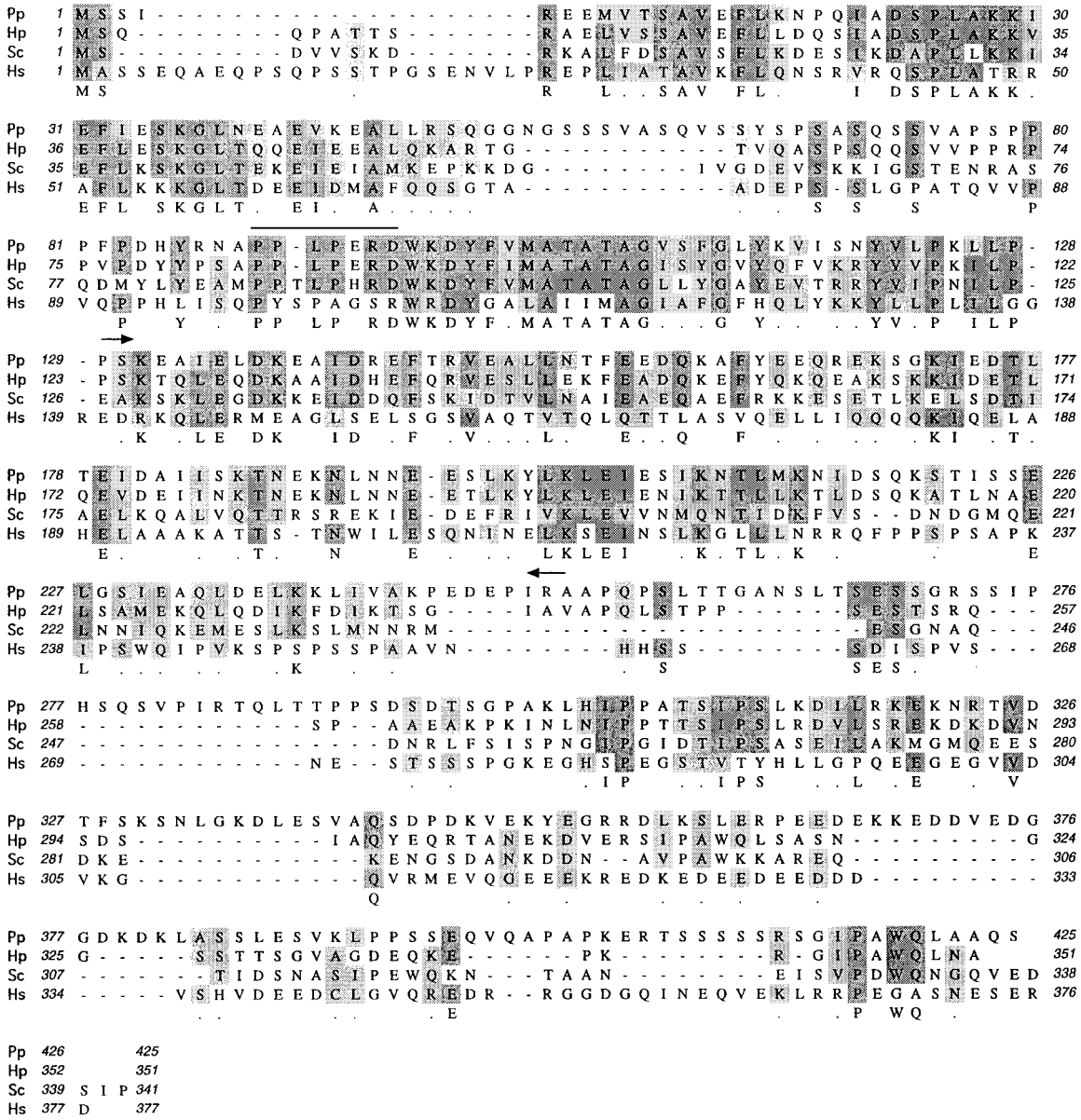


Figure 1. Alignment of the predicted amino acid sequences of Pex14p of *P. pastoris* (Pp), *H. polymorpha* (Hp), *S. cerevisiae* (Sc), and *H. sapiens* (Hs) using MacVector ClustalW. Amino acid residues identical in at least three sequences are shaded in dark grey, similar amino acids are shown in light grey. Similar residues are defined as follows: M=V=I=L; A=S=C; T=S=A; K=R=Q; N=T; E=D; E=Q; H=N; Q=H; N=G; F=Y. Hyphens represent spaces. Class II SH3–ligand binding domain is overlined. A predicted coiled-coil region is indicated between arrows. 1275 bp of *P. pastoris PEX14* sequence have been deposited in GenBank and were assigned Accession No. AF200421

To investigate the PTS1 import pathway in *pex14-1* and *pex14*Δ strains, methanol-induced cells were first assayed for AOX, a known PTS1 protein. As seen for other *P. pastoris pex* mutants, AOX activity in the mutants was negligible (Liu *et al.*, 1992; Johnson *et al.*, 1999) (data not shown). Next, subcellular fractions from both methanol- and oleate-induced mutant cells were assayed for activity of CAT, a putative PTS1 protein. CAT activity was found to be distributed approximately equally between the pellet and supernatant fractions of the wild-type strain (due to typical leakiness of

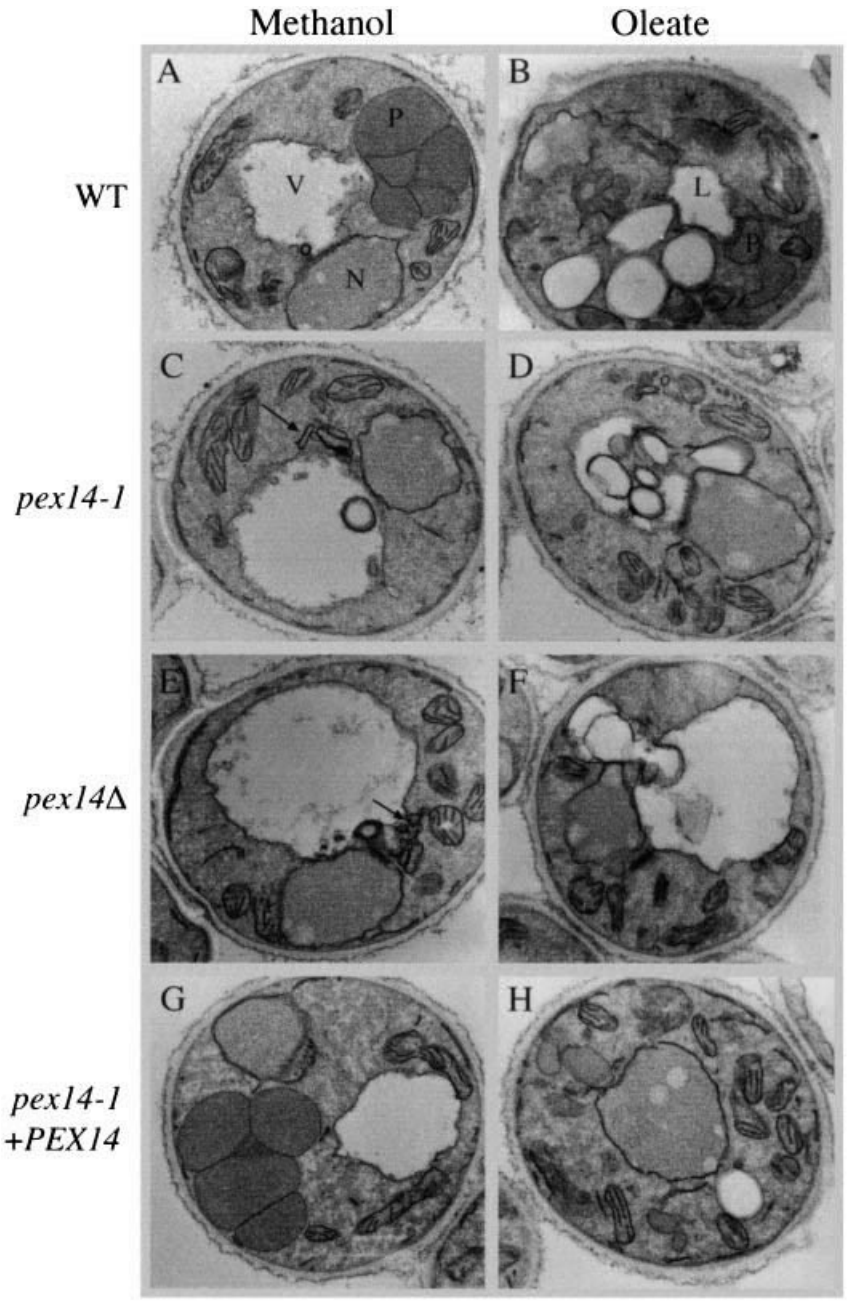
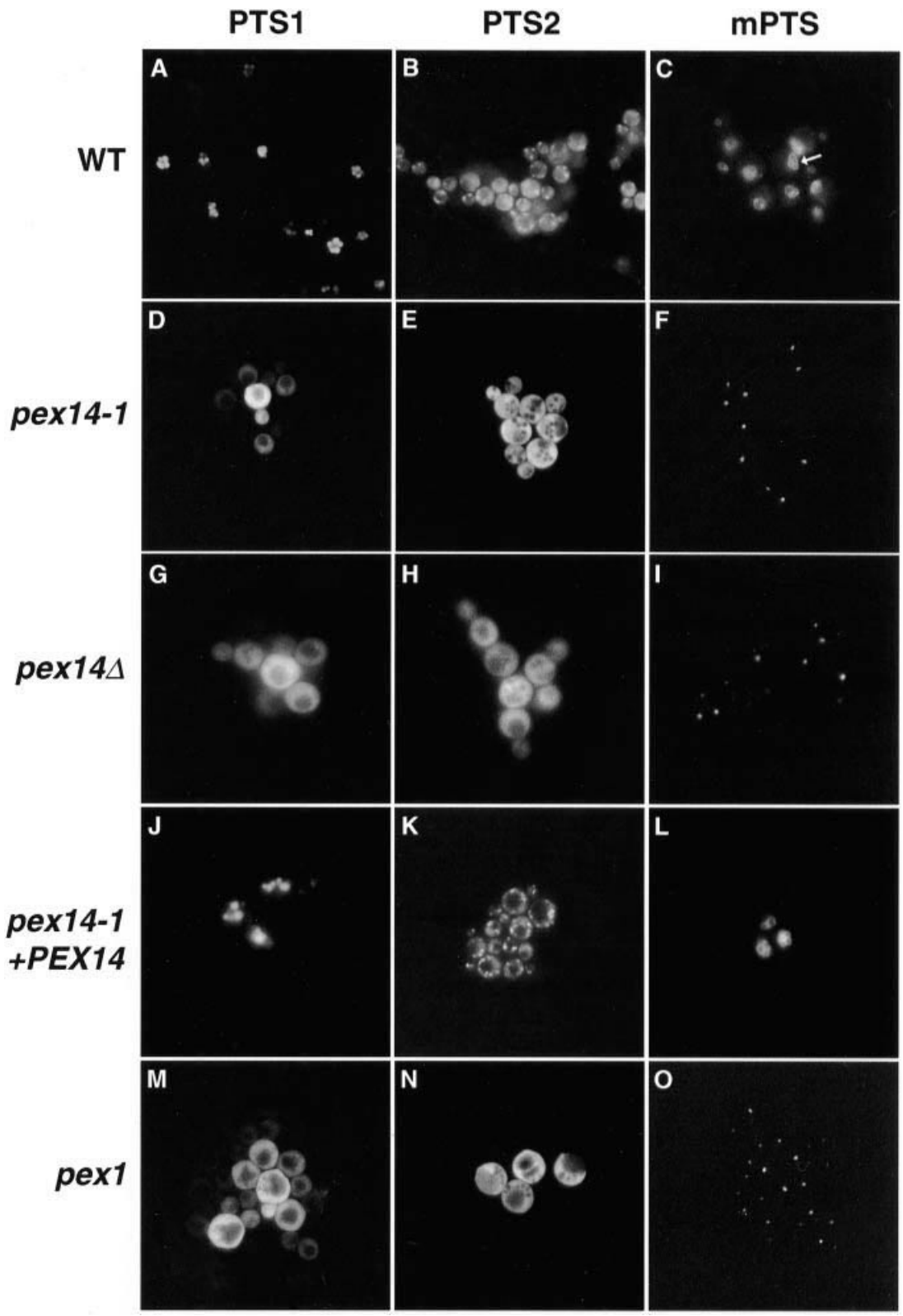


Figure 2. Electron micrographs showing subcellular morphology of wild-type (WT), *pex14-1*, *pex14*Δ, and *pex14-1* + *PEX14* (rescued) strains. (A) Proliferation of large clusters of peroxisomes in methanol-induced WT cells. (B) Proliferation of small or disperse peroxisomes in oleate-induced wild-type cells. (C, D) Lack of recognizable peroxisomes in *pex14-1* methanol- and oleate-induced cells, respectively (putative peroxisomal remnants indicated by arrow). (E, F) Lack of recognizable peroxisomes in *pex14*Δ strain induced in either methanol or oleate, respectively (putative peroxisomal remnants indicated by arrow). (G, H) Restored peroxisomes in methanol- and oleate-induced *PEX14* rescued cells, respectively. p, peroxisome; n, nucleus; v, vacuole; L, lipid body



peroxisomes) but was found almost entirely in the supernatant fractions of the mutants (Table 3) (methanol data not shown). To further investigate PTS1 import, the fate of two PTS1 reporter proteins was followed in the *pex14* mutants. One reporter was luciferase (Luc), a known PTS1 protein (Gould *et al*., 1987). The second was EGFP fused to a peptide ending in the PTS1 AKL (EGFP-PTS1) (Johnson *et al*., 1999). As shown in Table 3, Luc was mislocalized in oleate-induced *pex14* mutants. Similarly, EGFP–PTS1 was mislocalized in methanol-induced mutants (Figure 3D, G) but properly targeted to peroxisomes in the wild-type and rescued strains (Figure 3A, J). These experiments demonstrate that *P. pastoris pex14* mutants cannot import PTS1 proteins.

The PTS2 pathway was investigated in the same manner using subcellular fractions from oleate-induced *pex14* mutant cells by examining the fate of the PTS2 enzyme, thiolase, and a PTS2–EGFP fusion protein (Glover *et al*., 1994). Fractions immunoblotted for thiolase showed that it was mislocalized to the cytosolic supernatant of the *pex14* mutants (Figure 4). The *pex14*Δ mutant had very low levels of thiolase. In the wild-type and rescued strains, thiolase was found primarily in the pellet fractions. Similarly, fluorescence microscopy of oleate-induced cells showed PTS2–EGFP mislocalized to the cytoplasm in the *pex14-1* and *pex14*Δ strains (Figure 3E, H, respectively) while the PTS2–EGFP was localized to the peroxisomes in wild-type and rescued cells (Figure 3B, K). These data demonstrate that, as with PTS1 import, import of PTS2 proteins is defective in both *P. pastoris pex14* strains.

Finally, the functioning of the integral membrane protein targeting signal (mPTS) pathway was examined by fluorescence microscopy using strains expressing EGFP fused to Pex2p, a known peroxisomal integral membrane protein (Waterham *et al*., 1996; Johnson *et al*., 1999). As seen in Figure 3C, the mPTS–EGFP was targeted to the peroxisomal membrane in methanol-induced wild-type cells (note ring structures). In methanol-induced cells of the *pex14* mutants, mPTS–EGFP was targeted to the peroxisomal remnants (Figure 3F, I). These data suggest that Pex14p is not necessary for the proper targeting of mPTS-containing proteins.

Table 3. Subcellular fractionation of oleate-induced *P. pastoris pex14* cells

Strain	Fraction	Cytochrome c oxidase (%)	Catalase (%)	Luciferase (%)
WT	P	99	57	49
	S	1	43	51
pex14-1	P	95	1	1
	S	5	99	99
*pex14*Δ	P	96	2	9
	S	4	98	91
pex14-1 + PEX14	P	97	63	55
	S	3	37	45
pex1	P	95	5	1
	S	5	95	99

P, pellet; S, supernatant; WT, wild-type.

Figure 3. Subcellular location of EGFP–PTS1, PTS2–EGFP and mPTS–EGFP in wild-type (WT), *pex14-1*, *pex14*Δ and *PEX14* rescued strains. (A, D, G, J, M) Cells expressing PTS1–EGFP on methanol. Note the clusters of strongly fluorescing peroxisomes in WT (A) and rescued (J) strains vs. the cytosolic fluorescence in the *pex14-1* (D), *pex14*Δ (G), and *pex1* control (N) strains. (B, E, H, K, N) Cells expressing PTS2–EGFP on oleate. As with EGFP–PTS1 strains, the WT cells (B) and rescued cells (K) exhibited a punctate pattern, although this pattern was less pronounced than in A vs. the diffuse pattern seen in *pex14-1* (E), *pex14*Δ (H) and *pex1* (M) strains. (C, F, I, L, O) Cells expressing mPTS–EGFP on methanol. WT (C) and the rescued (L) strains exhibited localization of fluorescence to the peroxisomal membranes, seen as rings. Arrow denotes a cell in which rings are visible. *pex14-1* (F), *pex14*Δ (I) and *pex1* (O) show fluorescence localized to peroxisomal remnants

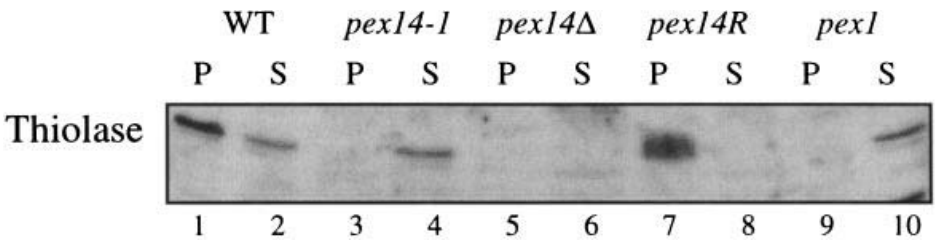


Figure 4. Subcellular localization of thiolase. Organelle pellet and cytosolic supernatant fractions obtained after subcellular fractionation of oleate-induced wild-type (WT), *pex14-1*, *pex14*Δ, *pex14* rescued (R), and *pex1* strains analysed by immunoblotting with antibodies against thiolase

Pex14p is tightly associated with the cytosolic surface of the peroxisomal membrane

To visualize Pex14p, polyclonal antibodies were raised in rabbits against the 146 C-terminal amino acids of Pex14p. Crude extracts prepared from wild-type *P. pastoris* cells and subjected to SDS–PAGE and immunoblotting showed a major band at approximately 58 kDa that was not present in extracts from the *pex14*Δ strain (see Figure 7A). Under SDS–PAGE conditions that maximized the separation of proteins in this size range, two bands reacted with the anti-Pex14p antibody: a major 58 kDa band that represented approximately 70% of Pex14p, and a slower-migrating band of approximately 60 kDa that represented about 30% of Pex14p. However, under standard SDS–PAGE conditions, these bands did not resolve and only one band was typically apparent (e.g. Figure 5C). The origin of the larger Pex14p species is discussed in the next section. Although both bands were visible regardless of carbon source, the overall level of Pex14p was greatest from lysates of wild-type cells induced on oleate (data not shown).

Pex14p localization in *P. pastoris* was investigated by first subjecting oleic acid-induced wild-type cells to subcellular fractionation. Pex14p was found primarily in the resulting pellet fraction (Figure 5A). To determine whether the Pex14p material in the pellet fraction was peroxisomal, pellet material was further fractionated by centrifugation through a sucrose-density gradient (Waterham *et al.*, 1996). Immunoblotting of fractions from this gradient with Pex14p and CAT antibodies showed that Pex14p co-sedimented with CAT, indicating that Pex14p is a peroxisomal protein (Figure 5B, C).

The nature of the association of Pex14p with the organelle was examined by subjecting the pellet from subcellular fractionations of oleate-induced wild-type cells to extraction with carbonate, pH 11.5. Pex14p was not extracted by these conditions, as observed by the presence of Pex14p in the post-extraction $100\,000 \times g$ pellet (Figure 6A). However, Pex14p was extracted by Triton X-100. As controls, a known peroxisomal integral membrane protein, Pex22p, also was not extracted from membranes by carbonate but CAT was extracted (Figure 6A). Thus, the behaviour of *P. pastoris* Pex14p was consistent with that of integral membrane proteins and, specifically, with that reported for mammalian and *H. polymorpha* Pex14ps (Komori *et al.*, 1997; Fransen *et al.*, 1998; Shimizu *et al.*, 1999; Will *et al.*, 1999) but not with that reported for *S. cerevisiae* Pex14p by Albertini and co-workers (1997), who found Pex14p fully extractable by carbonate at pH 11.5. In a subsequent report, Girzalsky *et al.* (1999) also observed that Pex14p was cytoplasmic in cells of a *S. cerevisiae* *pex13*Δ strain, further supporting the notion that, in *S. cerevisiae*, Pex14p is not an integral membrane protein but depends upon Pex13p, and perhaps other proteins, for its association with peroxisomal membranes. To examine the location of Pex14p in a *P. pastoris* *pex13*Δ strain, we subjected oleate-induced cells of the strain to subcellular fractionation and observed that *P. pastoris* Pex14p remained with the crude organellar pellet material (Figure 5A). This result indicated that, unlike the situation in *S. cerevisiae*, *P. pastoris* Pex14p remained with peroxisomal membranes in a *pex13*Δ strain.

The organellar pellets resulting from subcellular fractionation were incubated with increasing concentrations of trypsin. As seen in Figure 6B, Pex14p was sensitive to trypsin at the lowest concentration tested but remained stable in the

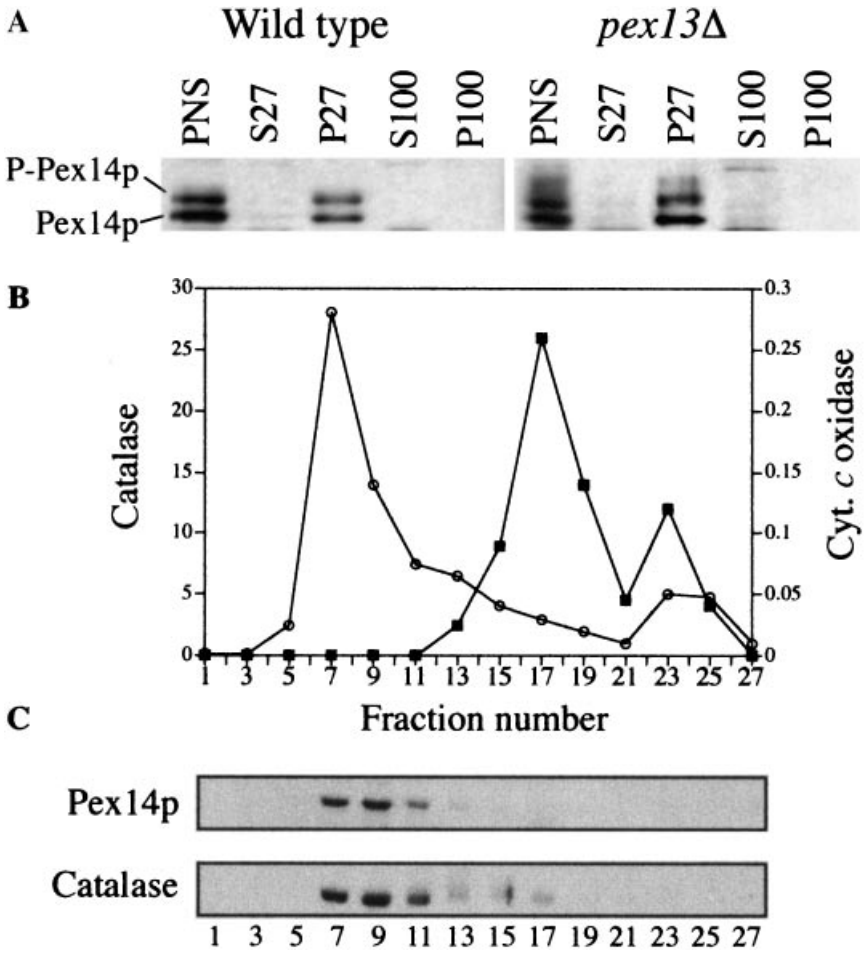


Figure 5. Pex14p is a peroxisomal protein. Subcellular fractionation of oleate-induced wild-type (A) and *pex13*Δ mutant (B) cells analysed by immunoblot with Pex14p antibodies. Fractions shown are: post-nuclear supernate (PNS), 27 000 × *g* supernate (S27) and pellet (P27), 100 000 × *g* supernate (S100) and pellet (P100). Equivalent portions of each fraction are shown. (C) Sucrose density gradient profile of the organellar pellet obtained from oleate-grown WT cells. Fractions collected from the gradient were assayed for peroxisomal CAT (○) and mitochondrial cytochrome *c* oxidase (■) activities. (D) Equal volumes of odd-numbered fractions of the sucrose density gradient in (B) were analysed by immunoblotting with the Pex14p and CAT antibodies

absence of trypsin. As a control, thiolase, a lumenal protein, was degraded by trypsin only if peroxisomes were disrupted through the addition of Triton X-100 (Figure 6B). Since the anti-Pex14p antibodies are directed at C-terminal residues (amino acids 146–425), these results indicate that at least the C-terminus of Pex14p is exposed on the cytoplasmic face of peroxisomes.

A portion of Pex14p is phosphorylated

Throughout our studies of Pex14p, we noted that careful separation of Pex14p through SDS–PAGE consistently revealed the peroxin as at least two distinct species: a major species at 58 kDa and a minor species at approximately 60 kDa. One explanation for the multiple bands was that the faster migrating species was a degradation product of the slower migrating species. To examine this possibility, we expressed the full *PEX14* ORF in *E. coli* and compared the size of the bacterial Pex14p to that of the *P. pastoris* wild-type cells. As seen in Figure 7B, only the faster migrating band was present in *E. coli* extracts. Because *E. coli* was unlikely to modify *P. pastoris* Pex14p, the

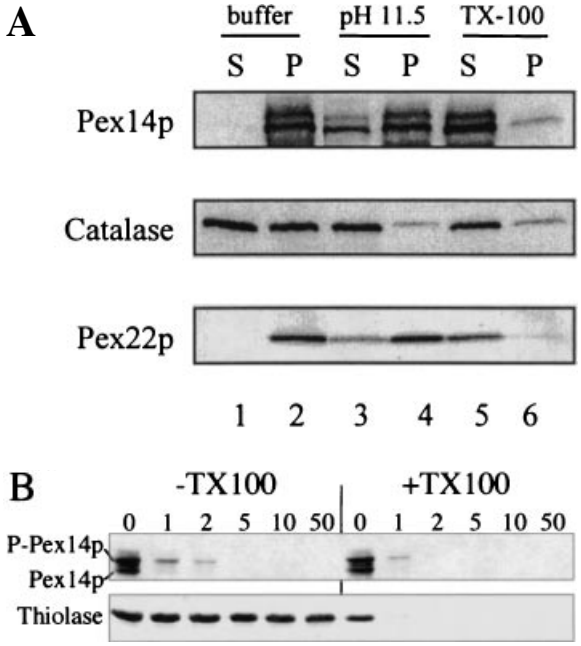


Figure 6. Membrane extraction and protease accessibility of Pex14p. (A) 1 mg total protein from cell lysates of oleate-grown wild-type (WT) cells was extracted with 0.1 M sodium carbonate, pH 11.5, centrifuged at 100 000 × *g*, and equal amounts of protein were loaded in each lane. The fractions were analysed by immunoblotting with Pex14p antibodies. (B) Aliquots of organellar pellet from oleate-induced WT cells were subjected to digestion with the specified amount of trypsin in the presence or absence of 0.1% Triton X-100. Equal volumes were subjected to SDS–PAGE and immunoblotted with either thiolase or Pex14p antibodies

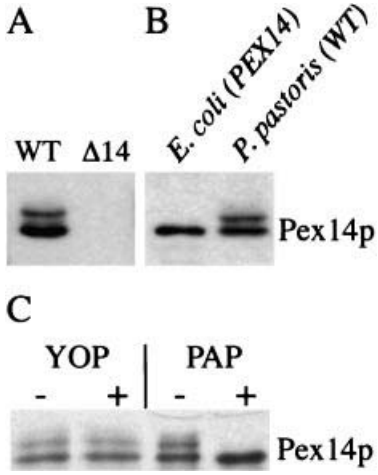


Figure 7. Pex14p is modified by phosphorylation. (A) Cell lysates from oleate-induced wild-type (WT) and *pex14*Δ were analysed by immunoblotting with Pex14p antibodies. (B) Approximately equal amounts of anti-Pex14p reacting material from extracts of an *E. coli PEX14* expression strain and a cell lysate from oleate-induced WT *P. pastoris* were analysed by immunoblotting with Pex14p antibodies. (C) *Yersinia* phosphatase (YOP) and potato acid phosphatase (PAP) treatment of *P. pastoris* extracts. Equal volumes of oleate-grown WT cell lysates were incubated with (+) and without (−) phosphatase and analysed by immunoblotting for Pex14p

bacterially synthesized 58 kDa protein most likely represented full-length unmodified Pex14p. This result suggested that the minor 60 kDa species may be a modified form of Pex14p.

We examined the possibility that the slower migrating band was a phosphorylated form of Pex14p. For this we treated wild-type oleate-induced cell lysates with the non-specific potato acid phosphatase (PAP) (Elgersma *et al.*, 1997) and the tyrosine-specific phosphatase from Yersinia (YOP) (Zhang *et al.*, 1992) (Figure 7C). PAP incubations resulted in the disappearance of the higher molecular mass species and a concomitant increase in the lower molecular mass species. In contrast, no change was observed upon treatment with YOP. We conclude that the larger species is a serine and/or threonine phosphorylated form of Pex14p.

Pex14p interacts With Pex3p, Pex5p, Pex7p, Pex8p, Pex13p, Pex17p and itself

In a previous study, we described the interaction between Pex14p and Pex3p (Snyder *et al.*, 1999b). To identify protein–protein interactions between Pex14p and other peroxins, the yeast two-hybrid system was used. When expressed as a DNA binding domain fusion, Pex14p interacted with Pex5p, Pex7p and Pex14p activation-domain fusions, as judged by transcriptional activation of both the *HIS3* and *LacZ* reporter genes (Figure 8A). The Pex14p DNA-binding domain did not, in concert with the empty activation domain, activate the reporter genes. Interaction was also observed between Pex14p in an activation-domain fusion with a cytosolic carboxy-terminal fragment (aa 55–267) of Pex17p (Figure 8B), while no activation was seen if the presumed lumenal and membrane-spanning region (aa 1–55) of Pex17p was the DNA-binding domain fusion partner. A Pex17p fragment composed of amino acids 1–142 was also

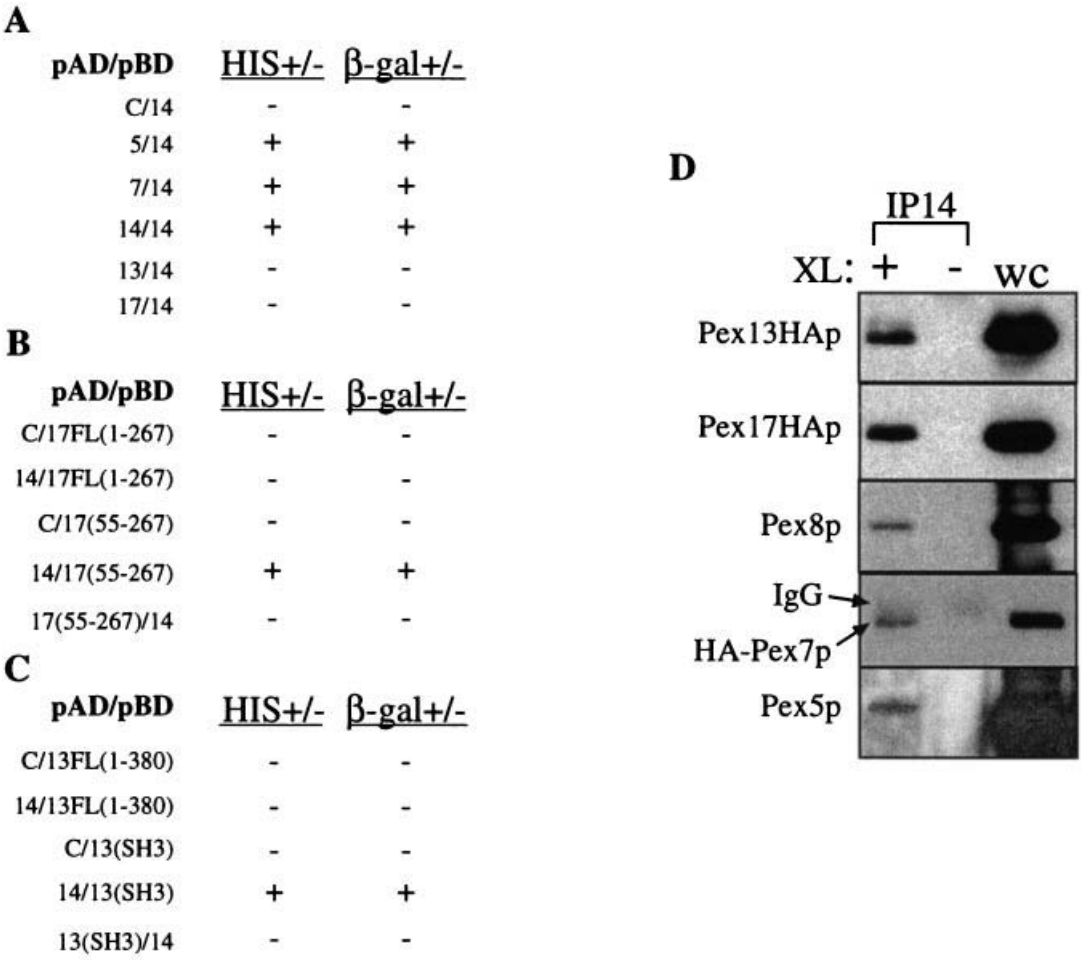


Figure 8. Interaction of Pex14p with multiple peroxins. (A, B, C) Two-hybrid analysis of interaction between Pex14p and Pex5p, Pex7p, Pex13p, Pex17p, and itself. The indicated hybrid protein constructs were tested for *trans*-activation of the *HIS3* gene, resulting in growth on medium lacking histidine, and *LacZ*, resulting in the production of β-galactosidase, as described in Materials and methods. Numbers refer to amino acids from Pex17p (B) or Pex13p (C). SH3, Src homology 3 domain of Pex13p; FL, full-length; pAD, transcriptional activation-domain fusion constructs; pBD, DNA binding domain fusion constructs; C, the presence of empty DNA binding or activation domain plasmids in the two-hybrid strains. (D) Crosslinking and co-immunoprecipitation of Pex5p, Pex7p, Pex8p, Pex13p and Pex17p with anti-Pex14p antibodies. Immunoprecipitations with affinity purified Pex14p antibodies from crosslinked (+) or non-crosslinked (−) extracts of oleate-grown cells expressing Pex8p, Pex5p, Pex13-HAp, Pex17-HAp or HA-Pex7p were analysed by immunoblotting. Pex13-HAp, Pex17-HAp and HA-Pex7p were immunoblotted with anti-HA. Pex5p and Pex8p were immunoblotted with anti-Pex5p and Pex8p antibodies, respectively. XL, crosslinker; IP14, immunoprecipitation with Pex14p antibodies. Whole-cell lysates (wc) were loaded (0.033A_{600}) as a control for immunoblotting. The amount of immunoprecipitation loaded (0.5 A_{600}) was 15-fold higher than in the wc lane

tested with Pex14p and resulted in no activation (data not shown). Thus, *P. pastoris* Pex14p (*Pp*Pex14p) interacts specifically with the carboxy-terminal fragment of Pex17p (aa 55–267).

A Pex14p activation-domain fusion also activated the two-hybrid system when paired with a DNA-binding domain fusion containing the SH3 region of Pex13p (Figure 8C), in agreement with published results (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Fransen *et al.*, 1998).

No two-hybrid interactions were seen between Pex14p and all other known *P. pastoris* peroxins (Pex1p, Pex2p, Pex4p, Pex6p, Pex10p, Pex12p, Pex19p and Pex22p).

The interactions identified using the two-hybrid system were confirmed by co-immunoprecipitation. HA-tagged versions of Pex17p, Pex13p and Pex7p were created and shown to fully complement *pex17*Δ, *pex13*Δ and *pex7*Δ mutant strains, respectively (data not shown). Extracts from oleate-induced cells expressing *PEX13-HA*, *PEX17-HA* and *HA-PEX7* were prepared, crosslinked with the cleavable crosslinker, DSP, and immunoprecipitated using the anti-Pex14p antisera. Following immunoprecipitation, a reducing agent was used to dissociate the crosslinked proteins. The immunoprecipitated materials were separated by SDS–PAGE and immunoblotted using monoclonal anti-HA antibodies or polyclonal antibodies against selected peroxins. As shown in Figure 8D, Pex13-HAp, Pex17-HAp, HA-Pex7p and Pex5p each co-immunoprecipitated with Pex14p. Interestingly, Pex8p was also detected as a member of the immunoprecipitation complex (Figure 8D). The crosslinking reaction appeared to be specific. We did not observe Pex2p or Pex19p in these complexes (data not shown), suggesting that our crosslinking procedure did not crosslink all peroxisomal proteins, a conclusion supported further supported by previous publications (Koller *et al.*, 1999; Snyder *et al.*, 1999b).

The presence of Pex8p in a complex with Pex14p had not previously been reported. Attempts to detect interaction between Pex14p and Pex8p via the two-hybrid system gave negative results. A Pex8p-binding domain fusion and a Pex14p-activating domain fusion, both of which were able to strongly activate the system with other two-hybrid partners, gave no response when paired with each other (data not shown and Figure 8A, B, C, respectively). It is clear from these results that Pex8p and Pex14p are components of the same complex but probably do not directly interact, hence the negative result by the two-hybrid system. These data suggest that Pex14p is in a complex with Pex3p, Pex5p, Pex7p, Pex8p, Pex13p, Pex17p and itself.

Both the phosphorylated and unmodified forms of Pex14p complex with Pex7p, Pex13p and Pex17p

We recognized that co-immunoprecipitation of the Pex14p complex components had the potential to provide an important insight into the function of phosphorylated Pex14p. However, these experiments were technically difficult, due to coincident molecular weights of Pex14p and IgGs. These IgGs from the immunoprecipitation gave a very strong signal in the immunoblots due to binding of the secondary antibody, anti-rabbit, thus masking the Pex14p. This masking problem was partially relieved by using a set of strains expressing HA-tagged versions of selected peroxins, since the HA antibodies were from a different species (mouse) than the anti-Pex14p antibodies (rabbit) used for blotting. Oleate-induced extracts of the HA-Pex7p-, Pex13-HAp- and Pex17-HAp-expressing strains were crosslinked and immunoprecipitated with HA antibodies. The precipitate was then subjected to SDS–PAGE and immunoblotting for Pex14p. To circumvent the IgG masking problem, we used goat anti-rabbit peroxidase-conjugated secondary antibodies pre-absorbed against mouse sera. By doing so, we reduced the amount of peroxidase-labelled secondary antibody binding to the IgGs from the immunoprecipitation, and we were able to clearly observe Pex14p. We found that both the phosphorylated and unphosphorylated forms of Pex14p immunoprecipitated with HA-Pex7p, Pex13-HAp and Pex17-HAp (Figure 9). Thus, complexes containing Pex14p appear to contain both forms of Pex14p in proportions similar to those observed for total Pex14p. Figure 10 schematically summarizes the Pex14p interactions we observed.

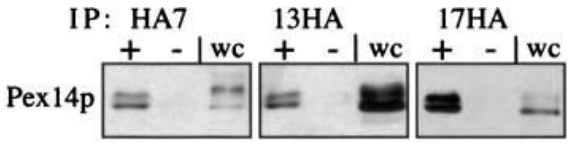


Figure 9. Immunoprecipitation of Pex14p with HA monoclonal antibodies in Pex17–HAp and Pex13–HAp expressing strains. Pex14p immunoprecipitated using HA antibodies and extracts prepared from the HA–Pex7p (HA7), Pex13–HAp (13HA) and Pex17–HAp (17A) expression strains. wc, Whole-cell extract; IP, immunoprecipitated material; XL, crosslinking; +, with crosslinker; −, without crosslinker. 0.5 A_{600} equivalents of each IP and 0.02 A_{600} equivalents of the whole cell lysate (wc) were loaded for 13HA and 17HA. For HA7, 0.002 A_{600} equivalents of the whole cell lysate (wc) were loaded

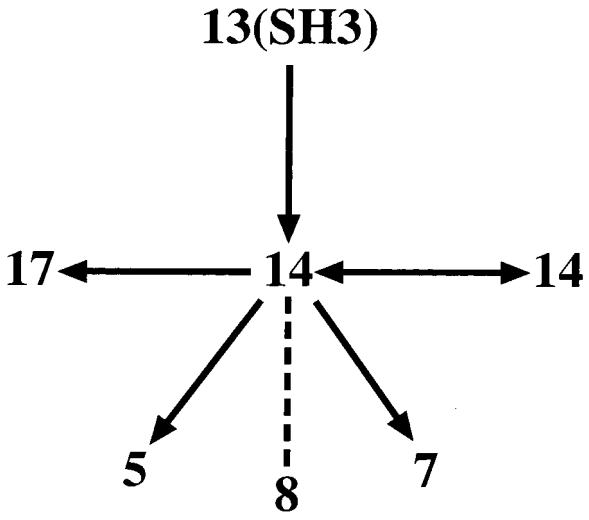


Figure 10. Schematic representation of interactions between Pex14p and other peroxins. Arrowheads represent peroxins in activation domain, and arrow tail represents peroxins in DNA binding domains in the two-hybrid constructions. Dashed line indicates that no two-hybrid interaction was observed. IP, immunoprecipitation; SH3, Src homology 3 domain

Discussion

This report describes the identification and characterization of the *P. pastoris* peroxin Pex14p, a protein essential for peroxisome biogenesis. Orthologues of this peroxin have been described in yeasts (*S. cerevisiae* and *H. polymorpha*) and mammals (humans, rats and CHO cells) (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Komori *et al.*, 1997; Fransen *et al.*, 1998; Shimizu *et al.*, 1999; Will *et al.*, 1999). The hypothesis that Pex14p serves an essential role in the import of peroxisomal matrix proteins as a receptor-docking protein is consistent with each of these previous reports and also the work presented here. The evidence for this function consists primarily of the observations that Pex14p is located on the surface of the peroxisomal membrane and that Pex14p directly interacts with the PTS receptors, Pex5p and Pex7p, and with several other peroxins (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Huhse *et al.*, 1998; Girzalsky *et al.*, 1999). Also consistent with a role for Pex14p in matrix protein import is the observation that the *pex14* mutants in each of these organisms are defective in the import of both PTS1 and PTS2 proteins, while the targeting of peroxisomal membrane proteins appears normal.

Comparison of Pex14p orthologues

*Pp*Pex14p shares these and other similarities with Pex14p from other species. As expected, the predicted amino acid sequence of *Pp*Pex14p is closest in similarity to those of the other yeast Pex14ps (29% identical and 41% similar to *Sc*Pex14p and 52% identical and 66% similar to *H. polymorpha* Pex14p) relative to those from mammals (15% identical and 22% similar to *Homo sapiens* Pex14p and 16% identical and 22% similar to CHO Pex14p) (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Komori *et al.*, 1997; Fransen *et al.*, 1998; Shimizu *et al.*, 1999; Will *et al.*, 1999). Interestingly, *Pp*Pex14p, at 425 aa, is 55–70 aa longer than the others. Alignment of the Pex14 polypeptides indicates that the 'extra' residues are primarily confined to the C-terminal region of *Pp*Pex14p, a region that is not strongly conserved among Pex14ps. The 'extra' amino acids include numerous charged residues but their significance, if any, is unknown.

The *Pp*Pex14p sequence contains potential secondary structures that are also present in other Pex14ps. All known Pex14p sequences predict the presence of a coiled-coil motif, shown in other proteins to be involved in oligomerization (Lupas *et al.*, 1991). Since most (all except human) Pex14ps, including *Pp*Pex14p, oligomerize (as judged by the yeast two-hybrid system results), the coiled-coil motif may play a role in their homo-oligomerization or oligomerization with other coiled-coil-containing proteins such as Pex17p, although this has not been directly demonstrated. A second structural motif that appears universal to yeast Pex14ps is a class II SH3-ligand motif, a ligand known to be bound by SH3 domains (Feng *et al.*, 1994). Such an SH3 domain exists in the yeast Pex13ps and has been shown both *in vivo* and *in vitro* to be important for binding of Pex14p to Pex13p in *S. cerevisiae* (Girzalsky *et al.*, 1999). In *S. cerevisiae,* one function of the SH3-ligand binding motif appears to be the tethering of Pex14p to the outer surface of the peroxisome through Pex13p. Here, we demonstrate that, although *P. pastoris* Pex13p also interacts with Pex14p through its SH3 domain, this interaction is not necessary for Pex14p localization. Interestingly,

the mammalian Pex14ps lack a class II SH3-ligand binding domain.

Subcellular location and topology of Pex14p

The nature of the association of Pex14p with the peroxisomal membrane was examined by multiple criteria. First, carbonate extraction experiments suggested that *Pp*Pex14p behaved as an integral membrane protein, consistent with what has been observed for mammalian and *H. polymorpha* Pex14ps. The situation in *S. cerevisiae* is more complicated. While the work of Brocard and co-workers (1997) suggested that *Sc*Pex14p was inextractable with carbonate, that of Albertini *et al.* (1997) reported that it was fully extractable. The criterion of carbonate inextractability has been used widely to determine whether a polypeptide is an integral membrane protein. However, this is an operational definition that should be supported by the presence of predicted transmembrane domains in the protein sequence, and inextractability could also arise by very tight interaction of a peripheral membrane protein with another integral membrane polypeptide. In this respect, *Pp*Pex14p interacts with two integral transmembrane proteins, *Pp*Pex13p and *Pp*Pex17p (see below). The clear definition of a predicted transmembrane domain in Pex14p would help clarify whether it is truly an integral membrane protein or simply behaves like one because of its tight association with other integral membrane proteins.

*Pp*Pex14p does not contain any sequence that looks like a normal transmembrane domain. As described in Results, only two of five transmembrane prediction programmes suggested the presence of a transmembrane domain in *Pp*Pex14p. That domain (aa 100–124) contains a few charged residues that are unlikely to be found in a hydrophobic environment. While this domain aligns to the predicted membrane-spanning region of mammalian Pex14ps, a single transmembrane segment is incompatible with the experimental data on the termini topology of rat Pex14p, as described below.

The localization of Pex14p to the peroxisome was investigated in *pex* mutant strains. In *S. cerevisiae*, Girzalsky and co-workers (1999) found that Pex14p was cytosolic in cells lacking Pex13p, suggesting that Pex13p might help anchor Pex14p on the peroxisomal membrane, in addition to its other functions. These results suggested that *Sc*Pex14p is not an integral membrane protein. We performed analogous experiments with *P. pastoris* and found that, in direct contrast to *Sc*Pex14p, *Pp*Pex14p remains associated with the peroxisomal remnants in a *P. pastoris pex13*Δ strain. This strongly suggests that Pex13p is not a determinant of Pex14p localization in *P. pastoris*. However, unlike *Sc*Pex17p, which behaves as a peripheral membrane protein that is extractable with carbonate, *Pp*Pex17p behaves as an integral membrane protein that is not extractable with carbonate. It is possible that the interaction of *Pp*Pex14p with *Pp*Pex17p in the *P. pastoris pex13*Δ stain would be sufficient to keep *Pp*Pex14p associated with the peroxisomal membrane, even if it is itself a peripheral protein. Therefore, these results do not clarify the nature of association of Pex14p with the membrane and may suggest that, in *P. pastoris*, association between Pex14p and Pex17p is more critical for Pex14p localization than its association with Pex13p. Accordingly, we tested the localization of Pex14p in a *pex17*Δ strain but found that it was not present and presumably had degraded (unpublished results).

Finally, the experimental observations regarding the cytosolic locations of both Pex14p termini are difficult to reconcile with the prediction that this protein spans the membrane. Since our anti-Pex14p antibodies are directed toward the C-terminus of *Pp*Pex14p, our trypsin digestion results support the notion that a significant portion of the *Pp*Pex14p C-terminus is orientated toward the cytoplasm. This has been observed previously in other organisms (Albertini *et al.*, 1997; Komori *et al.*, 1997; Shimizu *et al.*, 1999). However, Shimizu and co-workers (1999), using N- and C-terminally tagged versions of rat protein, provided evidence that both termini are exposed on the surface of the peroxisomal membrane. In addition, the protease accessibility of the C-terminus (see below) and an SH3-ligand motif (see above) in the N-terminal domain, which would interact with Pex13p in the cytosol, requires both of these regions to be cytosolic. Since the only predicted transmembrane domain is found between these regions, it is not possible for both regions to be cytosolic if the weakly predicted transmembrane domain is the only transmembrane region. Moreover, there do not appear to be two regions capable of spanning the membrane in Pex14p, which would be necessary to achieve the topology indicated by the work of Shimizu and co-workers (1999), and to

accommodate the protease protection data and the protein interaction data. Thus, the prediction of the membrane-spanning domain in Pex14p is speculative.

The only models consistent with all the data are those in which the hydrophobic region of Pex14p, which has a low probability to form a transmembrane domain, inserts into the membrane as a loop, rather than spanning the peroxisomal membrane, or Pex14p associates tightly with its partner proteins. Therefore, Pex14p would not be extractable with carbonate, and it could present its N- and C-terminal protein interaction domains to cytosolic binding partners. Until membrane-spanning domains are unambiguously identified in Pex14p, these models serve as reasonable hypotheses to explain its membrane-association properties.

Multiple peroxins exist in complexes with Pex14p

In *S. cerevisiae*, three other peroxins, in addition to Pex13p, have been identified as members of a Pex14p-containing matrix protein import complex. These proteins are Pex5p and Pex7p and the peripheral peroxisomal membrane protein Pex17p (Albertini *et al*., 1997; Brocard *et al*., 1997; Huhse *et al*., 1998; Girzalsky *et al*., 1999). By both two-hybrid and co-immunoprecipitation methods, we also observe these three peroxins in *Pp*Pex14p-containing complexes, but PpPex17p is an integral membrane protein. Additionally, we show for the first time that *Pp*Pex14p interacts specifically with the carboxy-terminal fragment of Pex17p (aa 57–267).

Our work (this paper and Snyder *et al*., 1999b) has defined two new members, Pex3p and Pex8p, as interacting partners with Pex14p. In co-immunoprecipitation experiments, the presence of Pex8p as part of a Pex14p-containing complex is consistently observed. However, we suspect that the interaction is indirect, since we did not observe the interaction between Pex8p and Pex14p via two-hybrid analysis. The presence of Pex8p and Pex14p in the same complex could be mediated by integral membrane proteins such as Pex13p, Pex17p and/or Pex3p (Snyder *et al*., 1999b), which we have defined as components of complexes containing Pex14p. Previously, a role for Pex8p in matrix protein import was established (Liu *et al*., 1995) and these new results reinforce that role with its identification as a member of the import machinery complex.

Cascade vs. complex activation models of docking protein function

In yeasts, a consensus picture is emerging in which a matrix protein import complex exists on the surface of the peroxisome. This complex is composed of directly interacting Pex13p and Pex14p, the latter as at least a homodimer, each of which interacts with Pex5p and Pex7p, although the possibility of indirect binding of Pex13p to Pex7p has not been entirely eliminated (Girzalsky *et al*., 1999). Pex17p appears to join the complex by direct interaction with Pex14p (Huhse *et al*., 1998; Snyder *et al*., 1999b), whereas Pex3p likely indirectly complexes with Pex14p. In addition to being a member of the matrix protein import complex, *P. pastoris* Pex17p appears to play a role in the insertion of integral membrane proteins (Snyder *et al*., 1999b).

In mammals (human, rat and CHO cells), the organization of this complex is not as well defined, with conflicting results obtained in different systems and/or with different techniques. Further studies are needed to determine whether the organization of this import complex actually varies between mammals or whether the observed differences are the consequence of the different techniques employed.

Setting aside the unresolved issues involving the putative mammalian import complex, how might this PTS-docking system work in the yeast import complex? Since Pex13p and Pex14p can bind both PTS receptors, the simplest model would be that Pex13p and Pex14p each perform the docking function independently of the other. However, this model also predicts that mutants defective in either Pex13p or Pex14p should be capable of at least some matrix protein import when, in fact, such mutants are completely defective in PTS1 and PTS2 protein import (Elgersma *et al*., 1996; Gould *et al*., 1996; Komori *et al*., 1997; Girzalsky *et al*., 1999). Two general models can accommodate this complication. One is a docking cascade model, in which a PTS receptor with its nascent protein cargo docks first with one specific docking protein (e.g. Pex14p) and then, in a second step, passes the PTS receptor–protein cargo on to Pex13p. When Pex17p binds to this complex poses another interesting question. Pex17p and Pex14p co-immunoprecipitate with both PTS receptors in the absence of Pex13p, suggesting that perhaps the complex of Pex14p, Pex5p or Pex7p, and Pex17p forms prior to binding with Pex13p (Huhse *et al*., 1998). The other model

is a single-step complex activation model, in which the PTS receptor plus cargo must simultaneously dock with a complex of both Pex13p and Pex14p, and perhaps Pex17p, to activate the next step in translocation.

We have discovered that *Pp*Pex14p exists in two forms. The majority of Pex14p (about 70%) is unmodified, while a portion of the protein (about 30%) is phosphorylated at one or more serine and/or threonine residues. During the preparation of this paper, Komori and co-workers published work showing that a portion ($\sim$50%) of *H. polymorpha* Pex14p is also phosphorylated (Komori *et al.*, 1999). Pex14p is the second phosphorylated peroxin described in yeasts after *S. cerevisiae* Pex15p, a peroxisomal integral membrane protein of unknown function (Elgersma *et al.*, 1997). Komori and co-workers (1999) observed that the relative amounts of modified and unmodified Pex14p forms seemed to vary with growth conditions in *H. polymorpha*. However, we do not see this in *P. pastoris*. The relative proportion of the two forms is not significantly different in glucose-, oleate- and methanol-grown cells, although the amount of both Pex14p forms increases significantly on the latter two peroxisome-inducing substrates (data not shown).

We examined whether the phosphorylation state of Pex14p affects its ability to associate in a complex (or complexes) with other peroxins, specifically Pex7p, Pex14p and Pex17p. However, none of these three peroxins showed a significant bias in co-immunoprecipitating the phosphorylated or unphosphorylated forms of the protein. For technical reasons, we were unable to extend these studies to Pex14p's other partners (i.e., Pex3p, Pex5p, Pex8p, and Pex14p itself). Thus, further studies are required to define the significance of Pex14p phosphorylation in peroxisomal protein import.

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