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The *Hansenula polymorpha PEX14* gene encodes a novel peroxisomal membrane protein essential for peroxisome biogenesis

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We have cloned the *Hansenula polymorpha PEX14* gene by functional complementation of the chemically induced *pex14-1* mutant, which lacked normal peroxisomes. The sequence of the *PEX14* gene predicts a novel protein product (Pex14p) of 39 kDa which showed no similarity to any known protein and lacked either of the two known peroxisomal targeting signals. Biochemical and electron microscopical analysis indicated that Pex14p is a component of the peroxisomal membrane. The synthesis of Pex14p is induced by peroxisome-inducing growth conditions. In cells of both *pex14-1* and a *PEX14* disruption mutant, peroxisomal membrane remnants were evident; these contained the *H.polymorpha* peroxisomal membrane protein Pex3p together with a small amount of the major peroxisomal matrix proteins alcohol oxidase, catalase and dihydroxyacetone synthase, the bulk of which resided in the cytosol. Unexpectedly, overproduction of Pex14p in wild-type *H.polymorpha* cells resulted in a peroxisome-deficient phenotype typified by the presence of numerous small vesicles which lacked matrix proteins; these were localized in the cytosol. Apparently, the stoichiometry of Pex14p relative to one or more other components of the peroxisome biogenesis machinery appears to be critical for protein import.

Keywords: *Hansenula polymorpha*/peroxisome/peroxisome deficiency/*PEX14* gene

Introduction

Microbodies (peroxisomes, glyoxysomes) are subcellular organelles found in virtually all eukaryotic cells (Subramani, 1993). Peroxisomes derive their name from the fact that the organelles contain enzymes involved in the generation and decomposition of H_2O_2, i.e. certain oxidases and catalase. However, the specific metabolic pathways vary greatly among the various organisms. For instance, in mammals, peroxisomes are not only involved in the β-oxidation of very long chain fatty acids, but also in the synthesis of cholesterol, bile acids, dolichol and etherphospholipids (van den Bosch *et al.*, 1992). The vital importance of peroxisomes in intermediary cell metabolism is illustrated convincingly in man, where peroxisomal dysfunctions (e.g. Zellweger syndrome) lead to severe abnormalities which are often lethal (Lazarow and Moser, 1989).

In the methylotrophic yeast *Hansenula polymorpha*, peroxisomes are involved in the metabolism of several growth substrates and are maximally induced during methylotrophic growth conditions (van der Klei *et al.*, 1991). Peroxisomes lack DNA and a protein-synthesizing machinery; their matrix proteins are synthesized in the cytosol on free polysomes and post-translationally imported into the target organelle. Two different peroxisomal targeting signals (PTSs) have been identified (Subramani, 1993; Rachubinski and Subramani, 1995); PTS1 is located at the extreme C-terminus and is characterized by the tripeptide sequence -SKL-COOH or conservative variants. PTS2 is located at the N-terminus and is characterized by the consensus sequence RL-X_5-H/QL. Both these signals and their translocation systems are conserved among lower and higher eukaryotes (Gould *et al.*, 1990; Gietl *et al.*, 1994). At present, an extensive effort directed at the unravelling of the molecular mechanism of peroxisome biogenesis and function is under way. Various genes essential for peroxisome biogenesis and function have been isolated using both forward and reversed genetic approaches (Kunau *et al.*, 1993). *Hansenula polymorpha* is an attractive model organism for such studies (van der Klei and Veenhuis, 1996). We have isolated and characterized a collection of peroxisome-deficient (*pex*) mutants of this organism (Titorenko *et al.*, 1993) and cloned 10 of the corresponding genes (van der Klei and Veenhuis, 1996).

Here, we describe the cloning of the *H.polymorpha PEX14* gene by functional complementation of a *pex14* mutant. We show that *PEX14* encodes a 39 kDa peroxisomal membrane protein that is essential for peroxisome biogenesis in *H.polymorpha* and appears to be involved in matrix protein import.

Results

Isolation and characterization of the PEX14 gene

The *H.polymorpha pex14-1* mutant is one of a collection of mutants, impaired for growth on methanol (Mut$^-$) (Titorenko *et al.*, 1993). Methanol-induced *pex14-1* cells invariably lack normal peroxisomes; instead these cells contained a large cytosolic crystalloid composed of alcohol oxidase (AOX) molecules (not shown). This phenotype is typical for chemical-induced *pex* mutants of *H.polymorpha* (van der Klei *et al.*, 1991). In addition, methanol-induced *pex14-1* cells characteristically contain peroxisomal remnants (Figure 1A). The *PEX14* gene was isolated by

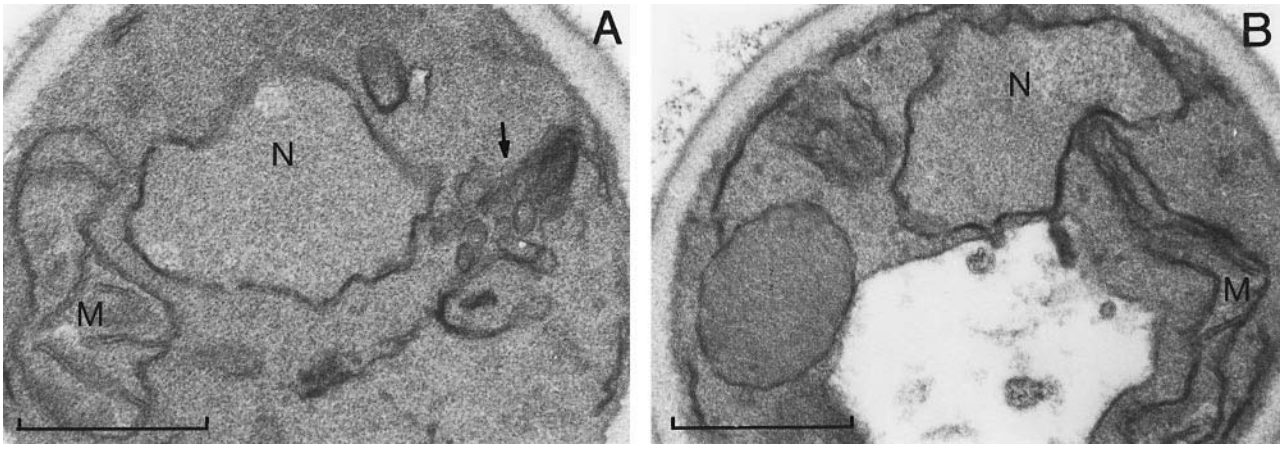

Fig. 1. Demonstration of peroxisomal remnants (arrow) in methanol-incubated cells of *pex14-1* (**A**). In the complemented strain, a peroxisomal profile is evident ($KMnO_4$; **B**). These and all subsequent electron micrographs are taken from glutaraldehyde-fixed cells, unless indicated otherwise. M, mitochondrion; N, nucleus. The scale bar represents 0.5 μm.

functional complementation of *pex14-1* using an *H.polymorpha* genomic library. Among ~3×10^4 Leu^+ transformants, one Mut^+ strain was observed. Upon retransformation of *pex14-1* cells with the plasmid (pPEX14-3) recovered from the Mut^+ transformant, again leucine prototropic cells were obtained which were capable of growth on methanol and contained morphologically normal peroxisomes (Figure 1B). The complementing plasmid contained an *H.polymorpha* DNA insert of 4.5 kb. By restriction and subcloning analysis, the complementing activity was found to reside on a 3.2 kb fragment, which subsequently was sequenced. The sequence was deposited at GenBank and was assigned the accession number U46195. The sequenced region appeared to contain three open reading frames (ORFs) with the potential to encode polypeptides of 351 (ORF351), 176 (ORF176) and 131 (ORF131) amino acids respectively, as well as a 75 bp DNA sequence with 80% homology to the *Saccharomyces cerevisiae* Asn-tRNA sequence (Figure 2; Biteau *et al.*, 1991). The latter sequence also included the Asn-tRNA anticodon GUU and therefore most probably represents an Asn-tRNA gene of *H.polymorpha*. Further analysis showed that a 1.5 kb *Dra*I–*Eco*RV subfragment that contained both ORF351 and ORF131 complemented the *pex14-1* mutant while a 0.6 kb *Xho*I–*Eco*RV subfragment that contained only ORF131 did not (Figure 2B). Furthermore, a DNA fragment in which a frameshift mutation had been introduced into ORF351 did not complement *pex14-1*. From this, we concluded that ORF351 contained the *pex14-1*-complementing activity.

Northern blot analysis, using the 1.5 kb *Dra*I–*Eco*RV fragment as probe, revealed a single transcript of ~1.3 kb in RNA extracted from fully derepressed wild-type cells grown in chemostat cultures on glucose/choline. The level of this transcript was much lower in glucose-grown cells (Figure 3). The size of the transcript and its inducibility in derepressed cells grown either on methanol or glucose/choline further supports the assumption that ORF351 is *PEX14*.

The *PEX14* gene encodes a polypeptide with a calculated mass of 39 kDa (Figure 4A). The deduced amino acid sequence had no significant overall similarity to any known protein in the databases, except for a weak similarity to two small regions in myosin heavy chains (amino acids

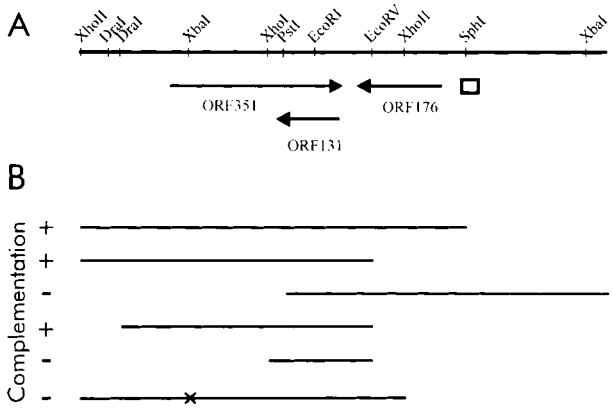

Fig. 2. (**A**) Schematic representation of the ORFs in the 3.2 kb *pex14-1*-complementing fragment. The putative *H.polymorpha* Asn-tRNA gene is indicated as a box. (**B**) Construction of various deletion fragments and their ability to complement the *pex14-1* mutant. The × marks a frameshift mutation.

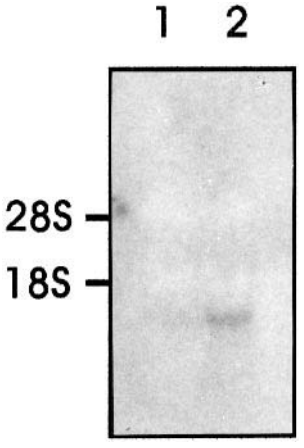

Fig. 3. Northern blot analysis of the induction of *PEX14* mRNA in wild-type cells. Total RNA (20 μg) from glucose-grown batch cells (lane 1) or glucose/choline-chemostat cells (lane 2) was used. The position of the yeast 18S and 28S rRNAs is indicated.

127–159 and 161–218; 35% identity; Cohen *et al.*, 1987). This similarity seems to be related to the presence of predicted coiled-coil regions in these proteins (Lupas *et al.*, 1991). Also, Pex14p lacked both of the two

A

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MSQQPATTSR AELVSSAVEF LLDQSIADSP LAKKVEFLES KGLTQQEIEE      50
ALQKARTGTV QASPSQQSVV PPRPPVPDYY PSAPPLPERD WKDYFIMATA     100
TAGISYGVYQ FVKRYVVPKI LPPSKTQLEQ DKAAIDHEFQ RVESLLEKFE     150
ADQKEFYQKQ EAKSKKIDET LQEVDEIINK TNEKNLNNEE TLKYLKLEIE     200
NIKTTLLKTL DSQKATLNAE LSAMEKQLQD IKFDIKTSGI AVAPQLSTPP     250
SESTSRQSPA AEAKPKINLN IPPTTSIPSL RDVLSREKDK DVNSDSIAQY     300
EQRTANEKDV ERSIPAWQLS ASNGGSSTTS GVAGDEQKEP KRGIPAWQLNA    351
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B

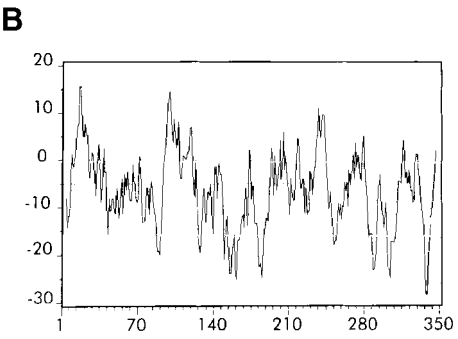

Fig. 4. (**A**) Predicted amino acid sequence of the *H.polymorpha PEX14* gene product. The weak homologous regions to myosin heavy chain and the potential coiled-coil regions are indicated as solid and dotted underlines, respectively. The potential phosphorylation (S or T) and myristylation (G) sites are indicated in bold italics. (**B**) Hydropathy profile of the predicted primary sequence of Pex14p. Computer analysis was done using the program SOAP or the PC-GENE package (an interval of nine amino acids).

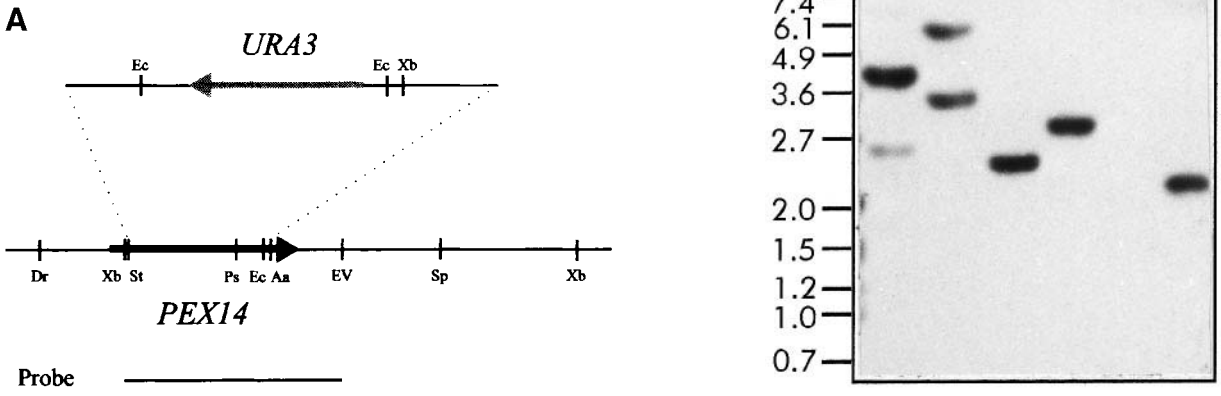

Fig. 5. Disruption of the *PEX14* gene. (**A**) Schematic representation of the deletion of the *PEX14* gene encoding most of the coding region (amino acids 43–329) by replacement with the *H.polymorpha URA3* gene. The disrupted *PEX14* gene was inserted into the wild-type *H.polymorpha* genome by homologous recombination. Aa, *Aat*II; Dr, *Dra*I; Ec, *Eco*RI; EV, *Eco*RV; Ps, *Pst*I; Sp, *Sph*I; St, *Stu*I; Xb, *Xba*I. (**B**) Correct integration of the Δ*pex14* fragment was demonstrated by Southern blot analysis. Chromosomal DNA (15 μg) isolated from wild-type (WT) and Δ*pex14* cells was digested with *Eco*RI, *Pst*I and *Xba*I. The 1.1 kb *Xba*I–*Eco*RV fragment of the *PEX14* gene was used as a probe.

conserved peroxisomal targeting signals (PTS1 and PTS2). Hydropathy analysis suggested that Pex14p contains one hydrophobic region located in the N-terminal region of the protein (amino acids 95–108), but lacks transmembrane domains (Figure 4B; Kyte and Doolittle, 1982).

Construction and characterization of a PEX14 deletion strain

An *H.polymorpha* strain was constructed in which most of the *PEX14* gene (the region encoding amino acids 43–329) was replaced by the *H.polymorpha URA3* gene (Figure 5A). After transformation of an *H.polymorpha leu1.1 ura3* host with the *pex14Δ::URA3* fragment, transformants were selected which were Ura^+ and Mut^-. Proper integration of the *pex14Δ::URA3* fragment into the *PEX14* genomic locus was confirmed by Southern blot analysis (Figure 5B). Like the original *pex14-1* mutant, the *pex14* deletion strain (Δ*pex14*) could not grow on methanol and lacked normal peroxisomes. The Δ*pex14* strain was crossed with a wild-type strain and the resulting diploids were sporulated and subjected to random spore analysis. As expected, Ura^+ and Mut^- phenotypes invariably co-segregated. Diploids resulting from crosses of the Δ*pex14* strain with the original *pex14-1* mutant strain displayed a Mut^- phenotype and lacked peroxisomes. Upon sporulation of these diploid strains, all spore progeny were Mut^- (122 segregants tested). These results demonstrate that the *pex14-1* and Δ*pex14* mutations are closely linked and most likely represent alleles of the same gene. From this, we concluded that we had cloned the authentic *PEX14* gene and not a suppressor gene.

Electron microscopical analysis of Δ*pex14* cells, incubated in batch cultures on methanol or grown in a carbon-limited chemostat on glucose/choline, revealed that the

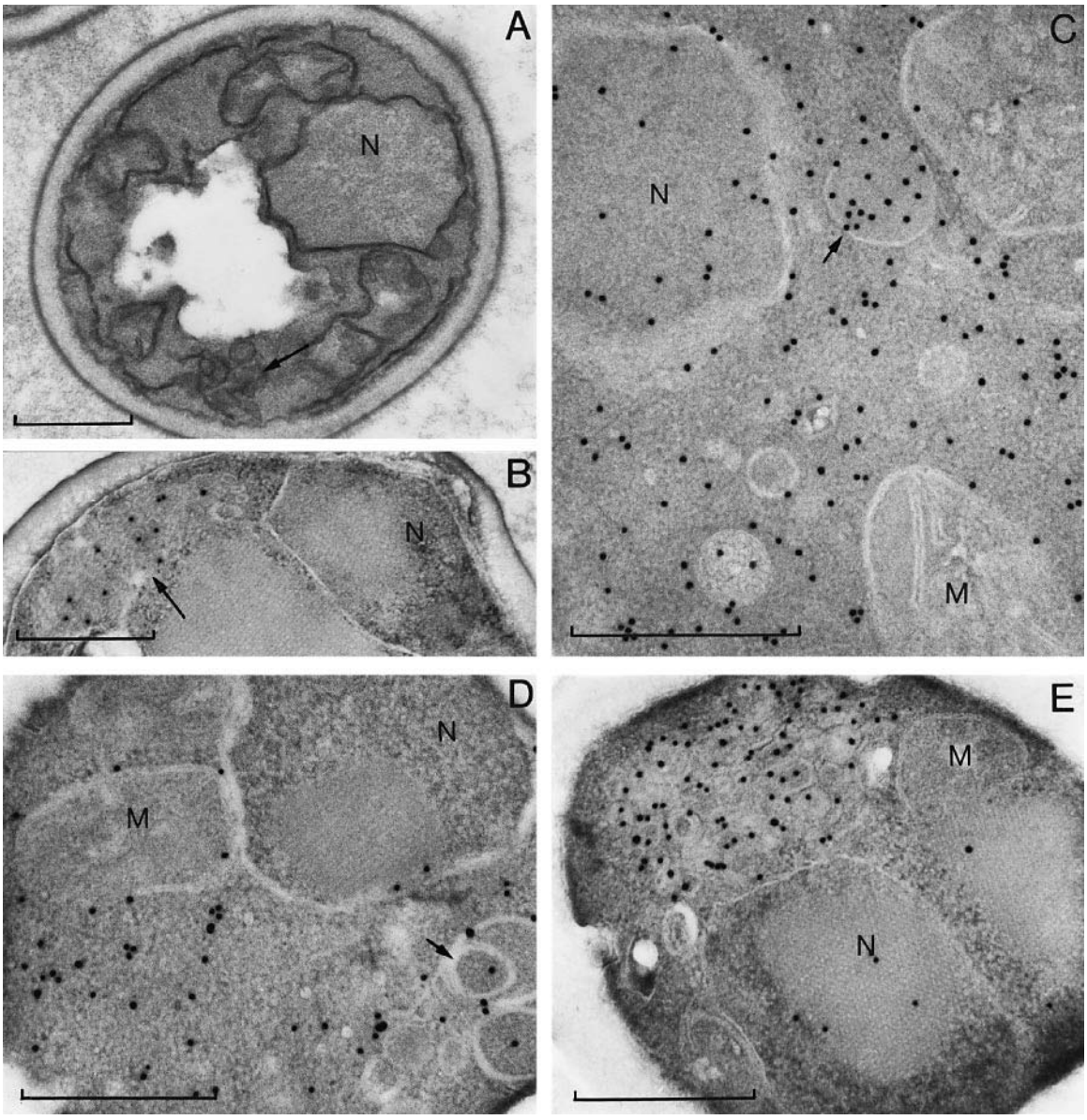

Fig. 6. Electron micrographs showing the morphology of Δ*pex14* cells. (**A**) The overall morphology of Δ*pex14* cells incubated in methanol-containing media. Peroxisomal membrane remnants are indicated by the arrow. The number of these vesicles is strongly enhanced in Δ*pex14* cells overproducing Pex10p (**B** and **E**). The vesicles are labelled in immunocytochemical experiments using α-Pex3p antibodies (B, arrow). Immunocytochemically, the bulk of the AOX (**C**) and CAT protein (**D**) is present in the cytosol and in the nucleus. In addition, labelling is observed in the vesicles (C and D; arrow). In Pex10p-overproducing Δ*pex14* cells, these membranes are proliferated and specifically labelled in experiments using α-Pex10p antibodies (E). (For key, see Figure 1.)

cells lacked intact peroxisomes but contained several small membranous vesicles (Figure 6A). Immunocytochemical experiments indicated that the major peroxisomal matrix proteins AOX (Figure 6C), catalase (CAT) (Figure 6D) and dihydroxyacetone synthase (DHAS, not shown) were localized in the cytosol and frequently also in the nucleus. In addition, a small but significant portion of AOX (Figure 6C), DHAS (not shown) and CAT labelling (Figure 6D) was associated with the membrane vesicles, indicating that these vesicles may represent peroxisomal membrane remnants (ghosts). The cytosolic localization of the major matrix proteins, including the PTS2 protein amine oxidase, was confirmed by Western blot analysis of the organellar pellets and soluble fractions obtained after differential centrifugation of crude cell homogenates (data not shown).

To verify further the peroxisomal nature of the membrane vesicles in the Δ*pex14* strain, Pex10p, a homologous *H.polymorpha* peroxisomal membrane protein, was over-produced in the strain. For this purpose, a plasmid that carries *PEX10* under the control of the *H.polymorpha* AOX promoter was transformed into the Δ*pex14* strain. As shown before (Veenhuis *et al.*, 1996), overproduction of Pex10p leads to the proliferation of the peroxisomal vesicles in Δ*pex* strains. In Δ*pex14*[P_{AOX}*PEX10*] cells this proliferation effect was indeed observed and Pex10p was sorted solely to these vesicles (Figure 6E). Since these membranes were also labelled in experiments using α-Pex3p antibodies (Figure 6B), we concluded that the vesicles, observed in the Δ*pex14* strain, indeed represent peroxisomal membrane remnants.

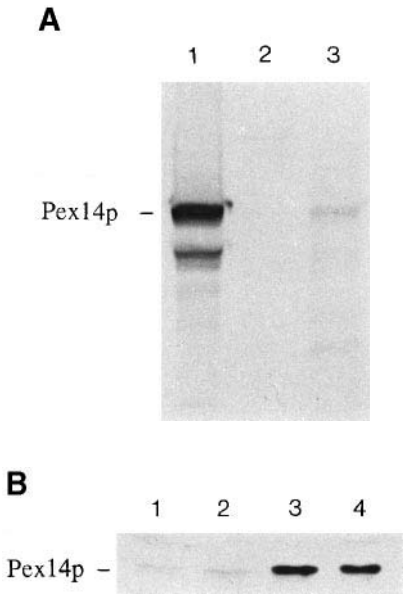

Fig. 7. (**A**) Western blots decorated with α-Pex14p antibodies to show the specificity of the antiserum. Lane 1, methanol-induced cells which overproduced Pex14p (P_{AOX}*PEX14*); lane 2, methanol-induced Δ*pex14* cells; lane 3, methanol-grown wild-type *H.polymorpha*. A 42 kDa band is recognized in extracts of the Pex14p-overproducing and the wild-type strain, which is absent in extracts of the Δ*pex14* strain. (**B**) Western blots decorated with α-Pex14p antibodies to demonstrate that Pex14p in induced by methanol but not by methylamine. Cells were grown in batch cultures and incubated for 18 h in methanol-containing media. Lane 1, glucose/ammonium sulfate; lane 2, glucose/methylamine; lane 3, methanol/ammonium sulfate; lane 4: methanol/methylamine (30 μg of protein were loaded per lane).

Subcellular localization of Pex14p

Polyclonal antibodies against Pex14p, generated in rabbit, were used for Western blot analysis of crude extracts prepared from variously grown cells. Blots prepared from cells overproducing Pex14p (wild-type[P_{AOX}*PEX14*]) showed a dominant protein band of ~42 kDa that was also present in extracts of methanol-grown wild-type cells, but absent in extracts of identically grown cells of the Δ*pex14* strain (Figure 7A). The electrophoretic mobility of Pex14p (42 kDa) was in good agreement with the calculated mass deduced from the amino acid sequence (39 kDa). A faint 42 kDa protein band was also detected in crude extracts of glucose-grown *H.polymorpha* wild-type cells (Figure 7B, lane 1). This band was enhanced significantly in blots prepared from extracts of methanol-grown wild-type cells (Figure 7B, lane 3) but appeared not to be induced further by the peroxisome-inducing nitrogen source methylamine, independently of the presence of methanol (Figure 7B, lanes 2 and 4). The subcellular localization of Pex14p was studied by conventional cell fractionation methods. After differential centrifugation of homogenates of methanol-grown wild-type cells, Pex14p sedimented in the 30 000 *g* organellar pellet and was absent in the 30 000 *g* supernatant fraction (Figure 8A). After subsequent sucrose density centrifugation of this organellar fraction, Pex14p co-sedimented with the peroxisomal marker proteins DHAS and Pex3p, indicating that Pex14p is a peroxisomal protein (Figure 8B). After high salt or sodium carbonate treatments of purified peroxisomal fractions, the major portion of Pex14p was pelletable; a comparable behaviour was observed for

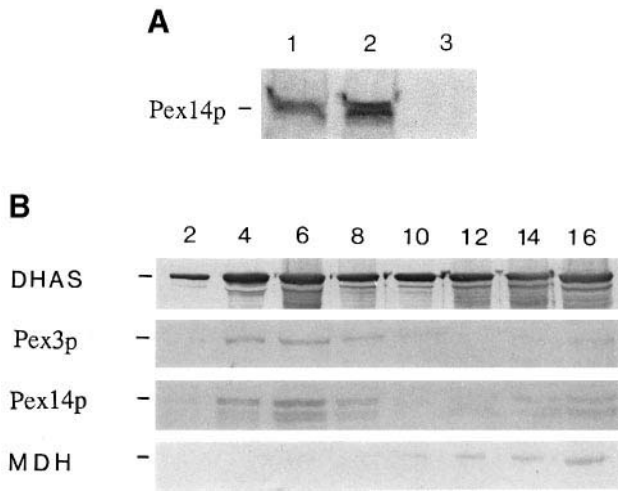

Fig. 8. (**A**) Western blots, decorated with α-Pex14p antibodies, prepared from the 30 000 *g* organellar pellet (lane 2) and 30 000 *g* supernatant (lane 3) obtained after differential centrifugation of homogenized protoplasts (lane 1) of methanol-grown wild-type *H.polymorpha* (30 μg of protein per lane). (**B**) The distribution of dihydroxyacetone synthase (DHAS), Pex14p, Pex3p and malate dehydrogenase (MDH) protein after sucrose density centrifugation of a 30 000 *g* organellar pellet, obtained from homogenized protoplasts. Fractions of 2 ml were collected from the bottom of the gradient. Equal volumes of the even numbered fractions were used for Western blotting and decorated with antibodies against Pex14p, the soluble peroxisomal matrix protein DHAS and mitochondrial MDH; the integral peroxisomal membrane protein Pex3p was used as control. Pex14p co-fractionates with the peroxisomal marker proteins in the high density fractions 4–8.

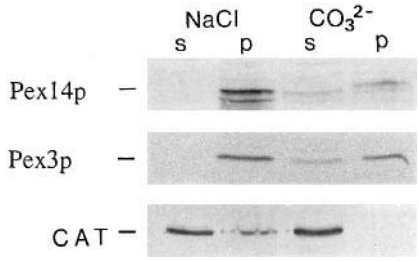

Fig. 9. Western blots demonstrating the distribution of Pex14p, CAT and Pex3p in the supernatant (S) and pellet fractions (P) after high salt (NaCl) or sodium carbonate treatment (CO_3^{2-}) of an organellar pellet. CAT was used as marker for soluble proteins, whereas Pex3p was used as peroxisomal membrane marker. Equal portions of the pellet and supernatant fractions were used.

Pex3p, an integral peroxisomal membrane protein (Baerends *et al.*, 1996), while CAT protein remained soluble (Figure 9). These data indicate that Pex14p is a component of the peroxisomal membrane of *H.polymorpha*. This location of Pex14p was confirmed immunocytochemically, using α-Pex14p antibodies (Figure 10A). Frequently, the α-Pex14p-specific labelling was found in one or a few clusters on the peroxisomal membranes (Figure 10A, inset). Pre-embedding labelling experiments revealed that Pex14p was accessible for antibodies which were added to purified intact organelles (data not shown), thereby suggesting that (at least part of) the polypeptide is exposed to the cytosol.

Pex14p overproduction results in peroxisome deficiency

We studied the effect of *PEX14* overexpression in wild-type cells carrying an additional copy of the *PEX14* gene

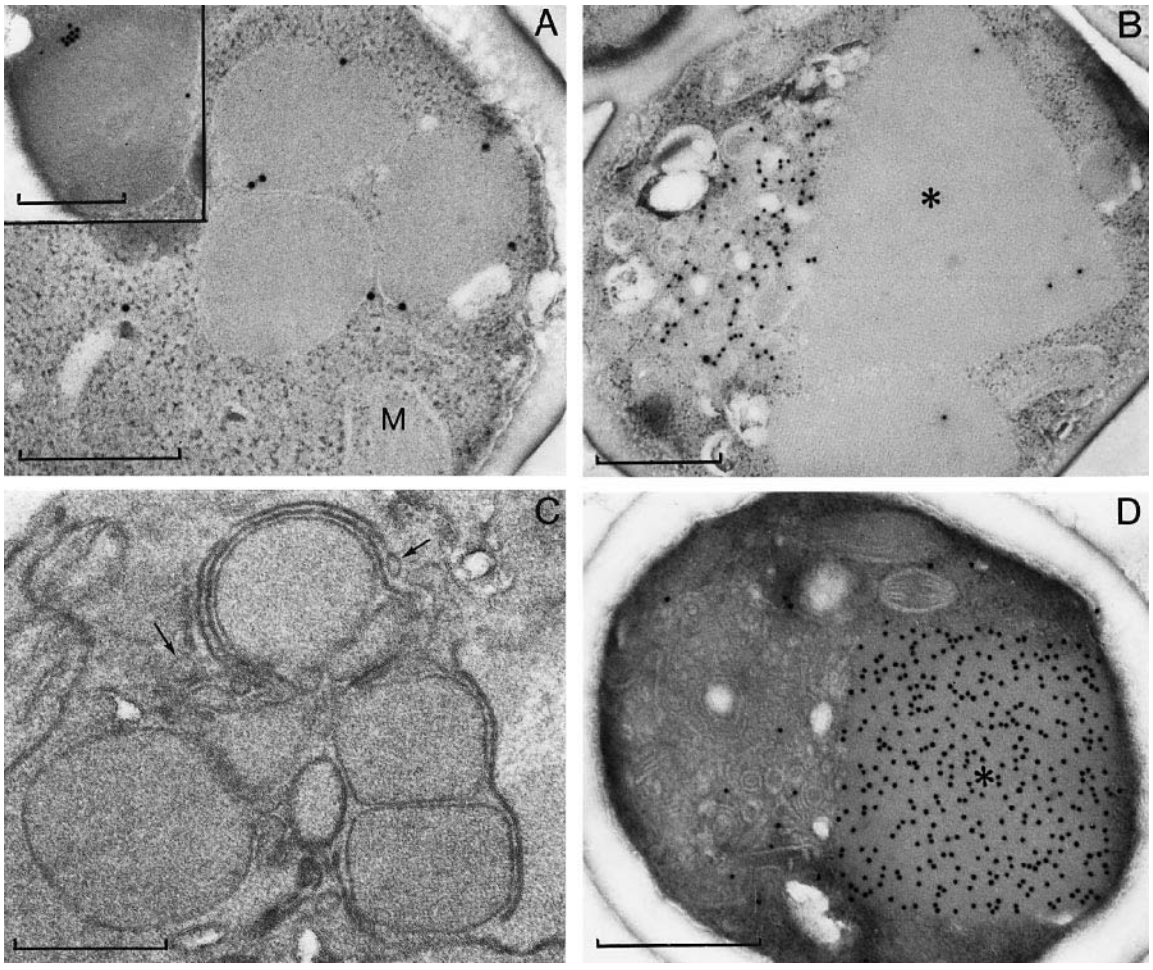

Fig. 10. Immunocytochemistry of methanol-grown wild-type cells using α-Pex14p antibodies. (**A**) Gold particles were found almost exclusively on the peroxisomal membranes and observed regularly in a cluster (inset). (**B**–**D**) The properties of cells from strains which artificially overexpress *PEX14*. Characteristic examples of wild-type[P_{AOX}*PEX14*] cells, showing the accumulation of membranous structures in the cells, are presented in (B) and (D). These structures are densely labelled after incubations of sections with α-Pex14p antibodies. Using antibodies against AOX, the crystalloids are labelled, but not the vesicles (D). At lower Pex14p levels, in Δ*pex14*[P_{AMO}*PEX14*] cells, peroxisomes are still present, associated with strands of endoplasmic reticulum-like membranes and small membranous vesicles (arrows in C). *, alcohol oxidase crystalloid. (For key, see Figure 1.)

under the control of the strong AOX promoter (P_{AOX}). Unexpectedly, this transformant was unable to grow in batch cultures supplemented with methanol as the sole carbon source. However, upon incubation of glucose-grown wild-type[P_{AOX}*PEX14*] in fresh methanol-containing media, Pex14p was induced rapidly. Ultrastructural analysis of these cells incubated for 18 h in methanol-containing media revealed that they lacked normal peroxisomes but contained numerous small vesicles in conjunction with a large cytosolic AOX crystalloid. The vesicles were always present in one cluster per cell (Figure 10B). Immunocytochemically, these structures were densely labelled when α-Pex14p antibodies were used, indicating that they contained Pex14p (Figure 10B). As expected, the crystalloids were labelled when α-AOX antibodies were used (Figure 10D); in the latter experiments, the vesicular regions were never labelled (Figure 10D). Identical results were obtained for DHAS and CAT protein (data not shown), indicating that the vesicles most probably do not contain major matrix proteins. These results indicated that Pex14p overproduction interfered with peroxisome biogenesis and/or matrix protein import. In order to determine the upper Pex14p levels which prescribe a wild-type peroxisomal phenotype, we re-introduced one copy of the *PEX14* gene, placed under control of either the strong methanol-inducible P_{AOX} or the weaker amine oxidase promoter (P_{AMO}), which is induced during growth of cells on primary amines, in a Δ*pex14* stain. In this way, the levels of Pex14p could be varied by manipulation of the growth conditions.

Cells of Δ*pex14*[P_{AOX}*PEX14*] grew normally on substrates which repress P_{AOX}, e.g. glucose. After a shift of glucose-grown cells to methanol-containing media, growth started, associated with the induction of Pex14p (Figure 11); newly formed peroxisomes were first detected after 3–4 h of incubation in the new growth environment. However, after further incubation, growth gradually ceased (final optical density OD_{663} = 0.8; OD_{663} wild-type controls = 3.1). Electron microscopical inspection of these cells revealed that their morphology was identical to wild-type cells expressing P_{AOX}*PEX14* in that they lacked peroxisomes, but were crowded with vesicles (data not shown; compare Figure 10B). In contrast, the growth patterns of Δ*pex14*[P_{AMO}*PEX14*] on methanol/methylamine were largely comparable with those of wild-type cells. Electron microscopical analyses of samples, taken

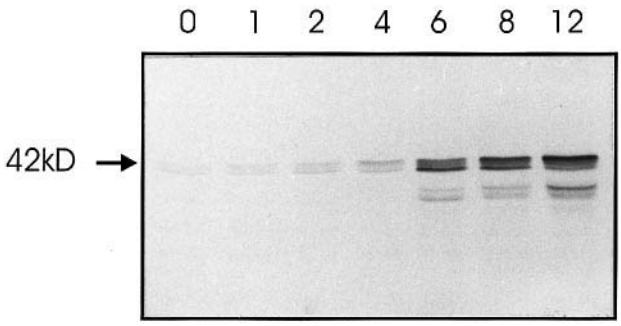

Fig. 11. Western blot analysis of crude extracts from Δ*pex14* cells containing P_{AOX}*PEX14*. Following the shift of cells from glucose to methanol-containing media, the synthesis of Pex14p is induced rapidly. A triple band is visible at 42 kDa. The protein bands with an apparent mol. wt of ~35 kDa most probably represent degradation products of Pex14p (compare also Figure 7A). Samples were taken at 1, 2, 4, 6, 8 and 12 h after the shift. Lanes were loaded with 20 µg of crude extract.

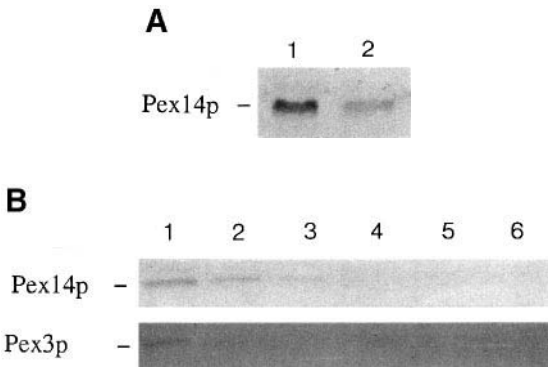

Fig. 12. (**A**) The distribution of Pex14p in the 30 000 *g* pellet fraction (P4, lane 1) and corresponding supernatant (S4, lane 2), obtained after differential centrifugation of homogenized, methanol-incubated wild-type[P_{AOX}*PEX14*] cells. Lanes were loaded with 20 µg of protein. (**B**) Flotation analysis of the 30 000 *g* pellet fraction (P4). After flotation centrifugation, Pex14p was detected in the top of the gradient (fractions 1 and 2), together with Pex3p. The blots were decorated with antisera against the proteins indicated (equal volumes were loaded per lane).

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MSQQPATTSRAELVSSAVEFLLDQSIADSPLAKKVEFLESKGLTQQEIEE -50
MSD-VVSKDRKALFDSAVSFLKDESIKDAPLLKKIEFLKSKGLTEKEIEI -49

ALQKARTGTVQASPSQQSVVPPRPPVPD----YYPSAPPLPERDWKDYFI -96
AMKEPKKDGIVGDEVSKKIGSTENRASQDMYLYEAMPPTLPHRDWKDYFV -99

MATATAGISYGVYQFVKRYVVPKILPPSKTQLEQDKAAIDHEFQRVESLL -146
MATATAGLLYGAYEVTRRYVIPNILPEAKSKLEGDKKEIDDQFSKIDTVL -149

EKFEADQKEFYQKQEAKSKKIDETLQEVDEIINKTNEKNLNNEETLKYLK -196
NAIEAEQAEFRKKESETLKELSDTIAELKQALVQTTRSREKIEDEFRIVK -199

LEIENIKTTLLKTLDSQKATLNAELSAMEKQLQDIKFDIKTSGIAVAPQL -246
LEVVNMQNTIDKFVSDNDGM--QELNNIQKEMESLK-SLMNNRMESGNAQ -246

STPPSESTSRQSPAAEAKPKINLNIPPTTSIPSLRDVLSREKDKDVNSDS -296
DNRLFSISPNGIPGIDTIPSASEILAKMGMQEESDKEKENGSDANKDDNA -296

IAQYEQRTANEKDVERSIPAWQLSASNGGSSTTSGVAGDEQKEPKRGIPA -346
VPAWKKAREQTIDSNASIPEWQKNTAANEISVPDWQNGQVEDS------- -339

WQLNA -351

---IP -341
```

Fig. 13. Amino acid sequence alignment of *H.polymorpha* (upper sequence) and *S.cerevisiae* (lower sequence) Pex14p. Identical residues are indicated by bars, similar ones by a dot. Gaps are introduced to maximize the similarity.

at different time intervals after the transfer of cells from glucose/ammonium sulfate to methanol/methylamine-containing media, revealed that the intermediate stage between normal wild-type peroxisome formation and aberrant peroxisome assembly was reached in the late exponential growth phase of the culture on methanol/methylamine; these cells characteristically contained enhanced numbers of peroxisomes associated with several vesicles and, infrequently, with strands of endoplasmic reticulum-like membranes (Figure 10C). Densitometric scanning of Western blots revealed that in these cells the Pex14p levels exceeded the values detected in wild-type controls ~4-fold.

We have purified the vesicles, present in wild-type[P_{AOX}*PEX14*] cells, harvested after 24 h of incubation, by differential centrifugation of homogenized protoplasts prepared from these cells. Western blot experiments revealed that Pex14p was pelletable and accumulated in the 30 000 *g* pellet (P4); attempts to purify the vesicles further by sucrose density centrifugation (the convential method for peroxisome purication) failed, since the vesicles largely remained in the overlay (data not shown). For this reason, the P4 fraction was analysed further by flotation centrifugation. Western blot analysis of the fractions obtained by this procedure revealed that Pex14p co-fractionated with Pex3p, a peroxisomal membrane protein of *H.polymorpha*, in fractions 1 and 2 (Figure 12); AOX was absent in these fractions. Other membrane proteins tested, e.g. Pex8p and Pex10p, were also absent or below the limit of detection (data not shown). Since AOX protein was also not detectable by immunocytochemical means (Figure 10D), we assume that the Pex14p-induced vesicles do contain peroxisomal membrane proteins, but lack matrix proteins.

Discussion

The chemically induced *H.polymorpha pex14-1* strain was one of a series of *H.polymorpha* mutants affected in peroxisome biogenesis (Titorenko *et al.*, 1993). In this report, we describe the cloning and characterization of the corresponding gene, *PEX14*. The *PEX14* gene encodes a novel polypeptide of 351 amino acids (39 kDa) with no overall sequence similarity to any other protein in the databases. However, a recent search of DNA sequences submitted as part of the *S.cerevisiae* genome sequencing project (EMBL accession No. Z48618, nt 25 583–26 608) revealed what may represent the bakers yeast homologue of *PEX14* (29% identity, 42% similarity). The N-terminal half of the proteins is particularly well conserved (amino acids 10–51 and 84–109; Figure 13). This part of the proteins contains hydrophobic regions which do not constitute membrane-spanning α-helices but may have an important role in the function of Pex14p. Genetic evidence indicated that the *PEX14* gene product is one of the five core components of peroxisome biogenesis in *H.polymorpha* and interacts with other peroxins (Titorenko *et al.*, 1993). Probably, these hydrophobic regions facilitate one or more of these interactions.

The Δ*pex14* mutant lacks intact peroxisomes but contains peroxisomal membrane remnants (vesicles). Similar vesicular structures have been observed in other *H.poly-*

Table I. Strains and plasmids used in this study

Strains/plasmids	Relevant properties	Source or reference
H.polymorpha		
pex14-1	*leu1.1 pex14* (originally named *per10-108*)	Titorenko *et al.* (1993)
pex14::URA3	*leu1.1 ura3 pex14::URA3*	this study
CBS4732	wild-type	CBS collection, The Netherlands
NCYC495	*leu1.1 ura3*	Gleeson and Sudbery (1988)
E.coli		
XL1-Blue MRF′	*Δ(mcrA)183Δ(mcr(B-hsdSMR-mrr)173endA1 supE44 thi-1recAgyrA96relA1lac [F′proABlacI^{q}ZΔM15Tn10(tet^r)]*	Stratagene
DH5α	*supE44ΔlacU169(ϕ80lacZΔM15)hsdR17recA1 endA1gyrA96 thi-1relA1*	Sambrook *et al*. (1989)
Plasmids		
pYT3	Ap^r, *LEU2, HARS1*	Tan *et al.* (1995)
pHIPX4-B	Km^r, *LEU2*, P$_{AOX}$	this study
pHIPX5	Km^r, *LEU2*, P$_{AMO}$	Kiel *et al.* (1995)
pBluescript II SK+	Ap^r	Stratagene
pMAL-c2	Ap^r, P$_{tac}$*mal*E	New England Biolabs
pET4	Km^r, *LEU2*, P$_{AOX}$*PEX10*	Tan *et al.* (1995)
pPEX14-3	Ap^r, *LEU2*, *HARS1*, 4.5 kb-*PEX14*	this study
pBS3.2P14	Ap^r, 3.2 kb-*PEX14*	this study
pBS1.5P14	Ap^r, 1.5 kb-*PEX14*	this study
pBS1.3P14	Ap^r, 1.3 kb-*PEX14*	this study
pP$_{AOX}$-PEX14	Km^r, *LEU2*, P$_{AOX}$*PEX14*	this study
pP$_{AMO}$-PEX14	Km^r, *LEU2*, P$_{AMO}$*PEX14*	this study

morpha pex mutants (*pex1*, *pex6* and *pex8*; Waterham *et al.*, 1994, Veenhuis *et al.*, 1996), the *Pichia pastoris* mutants *pex1* (Heyman *et al.*, 1994), *pex2* (Waterham *et al.*, 1996), *pex6* (Spong and Subramani, 1993) and *pex8* (Liu *et al.*, 1995), and *Yarrowia lipolytica pex9* (Eitzen *et al.*, 1995). In *H.polymorpha* Δ*pex14* cells, these vesicles contained peroxisomal membrane proteins (e.g. Pex10p and Pex3p) indicating (i) that they are peroxisomal in origin and (ii) that the peroxisomal membrane-synthesizing machinery (including sorting of the peroxisomal membrane proteins) is still operative in the mutant. This observation, together with the immunocytochemical demonstration that a very low amount of the major peroxisomal matrix proteins is present in these vesicles, suggests that *pex14* mutants are defective in matrix protein import and, therefore, that Pex14p is a component of the matrix protein import machinery.

The predicted amino acid sequence of Pex14p includes several potential phosphorylation and myristylation sites (see Figure 4A). The Pex14p triplet of bands at ~42 kDa seen in Western blots of crude extracts from cells overexpressing *PEX14* may, therefore, reflect different modified forms of the protein, although we cannot exclude that these bands represent degradation products. It is conceivable that Pex14p activity is regulated by changes in its phosphorylation state brought about by a kinase that responds to specific culture conditions. However, further investigation on this topic is required. Also possible myristylation of Pex14p would explain why this protein behaves like a membrane protein in spite of the fact that its sequence lacks predicted membrane-spanning regions (MSRs). However, the absence of an MSR in integral membrane proteins is not unique; an example is also found among the *H.polymorpha PEX* products, namely Pex10p, which is an integral membrane protein but has no apparent α-helical transmembrane domain (Tan *et al.*, 1995).

Overproduction of Pex14p changes the phenotype of *H.polymorpha* cells dramatically from wild-type to Per$^-$ again (i.e. Mut$^-$ and absence of peroxisomes); a major difference between the peroxisomal remnants in Δ*pex14* cells and the vesicles, contained in Pex14p-overproducing cells, is that the latter most probably do not contain matrix proteins. This result suggests that overproduction of Pex14p may either deplete other protein factor(s) essential for peroxisome biogenesis/matrix protein import or disturb the stoichiometry of these proteins (van der Klei and Veenhuis, 1996), which is essential for normal functioning. This is consistent with our view that protein interactions, probably mediated by the putative protein interaction domains (hydrophobic regions, coiled-coil structure), may play a vital role in Pex14p function(s). It is also in line with previous genetic studies that indicated that the *PEX14* gene product functionally interacts with the *PEX1* and *PEX6* gene products (Titorenko *et al.*, 1993), both belonging to the family of AAA-ATPases (Kunau *et al.*, 1993). Further studies to identify the specific function of Pex14p and to elucidate the protein(s) that functionally interact with Pex14p are underway.

Materials and methods

Organisms, media and growth conditions

Hansenula polymorpha and *Escherichia coli* strains and plasmids used in this study are listed in Table I. *H.polymorpha* was grown at 37°C in (i) rich medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 1% (w/v) glucose (YPD), (ii) selective minimal media containing 0.67% (w/v) yeast nitrogen base supplemented with 1% (w/v) glucose (YND) or 0.5% (v/v) methanol (YNM), or (iii) mineral medium supplemented with 0.5% (w/v) carbon source and 0.25% (w/v) nitrogen source (van Dijken *et al.*, 1976). Carbon-limited continuous cultures were grown on 0.25% (w/v) glucose and 0.2% (w/v) choline at a dilution rate of 0.06/h. Amino acids and uracil were added to a final concentration of 30 μg/ml. The *E.coli* strains were grown at 37°C in LB medium supplemented with the appropriate antibiotics.

Cloning and sequence analysis of the PEX14 gene

To isolate the *PEX14* gene, the *H.polymorpha pex14-1* mutant (Titorenko *et al.*, 1993) was electrotransformed (Faber *et al.*, 1994) with a *H.polymorpha* genomic DNA library constructed in vector pYT3 (Tan *et al.*, 1995). Leucine prototrophs were replica-plated onto YNM plates and screened for the ability to grow on methanol (Mut^+). Plasmid DNA was recovered from Mut^+ cells by electrotransformation of *E.coli* XL1-Blue MRF′. A plasmid containing an insert of ~4.5 kb was recovered and was analysed further. By subcloning, a 3.2 kb fragment was identified that complemented *pex14-1*. This fragment was inserted in both orientations into *Sma*I-digested pBluescript II SK^+ (Stratagene, San Diego, CA) to create plasmids pBS3.2P14a and pBS3.2P14b, and a series of nested deletions was generated by the limited exonuclease III digestion method (Sambrook *et al.*, 1989). Double-stranded DNA sequencing of the resulting subclones was carried out on an ABI 313A automatic sequencer (Applied Biosystems Inc.) using the Taq Dye Deoxy Terminator Cycle Sequencing Kit. In addition, several selected oligonucleotides were synthesized to complete or confirm certain portions of the DNA sequence. A frameshift was introduced into the *PEX14* ORF by cutting with *Xba*I, filling in the termini with Klenow and religating. This resulted in the introduction of a new stop codon just downstream of the destroyed *Xba*I site. For analysis of the DNA and amino acid sequences, the TBLASTN algorithm (Altschul *et al.*, 1990), the PC-GENE program release 6.70 (IntelliGenetic Inc., Mountain View, CA) and the GenBank (R) database Release 88.0 were used. Standard recombinant DNA techniques were performed as described (Sambrook *et al.*, 1989). Northern blot analysis was performed using a ^{32}P-labelled probe as described (Sambrook *et al.*, 1989).

Construction of a PEX14 deletion mutant

To disrupt the wild-type *PEX14* gene, a 2.3 kb *Bam*HI fragment (blunted by Klenow treatment) containing the *H.polymorpha URA3* gene (Merckelbach *et al.*, 1993) was ligated into *Stu*I and *Aat*II (blunt-ended)-digested pBS3.2P14a. This resulted in a deletion of an 862 bp region of the *PEX14* ORF encoding amino acids 43–329. The resulting plasmid was digested by *Dra*I and *Sph*I to yield a 3.5 kb linear fragment containing the *URA3* gene flanked by *PEX14* sequences and transformed to *H.polymorpha* NCYC 495 *leu1.1 ura3*. Uracil prototrophic transformants were selected and screened for the ability to utilize methanol. Methanol utilization-defective (Mut^-) strains were examined for the proper integration of the *URA3* gene into the *PEX14* locus of the genome by Southern blot analysis using the ECL direct nucleic acid labelling and detection system (Amersham Corp., Arlington Heights, IL). The resulting putative Δ*pex14* strains were also tested for complementation by the *pex14* complementing fragments. Segregation, complementation and linkage analyses of the Δ*pex14* mutant were performed as described previously (Titorenko *et al.*, 1993). To study the localization of peroxisomal membrane proteins in Δ*pex14* cells, Δ*pex14* was transformed with pET4 which contains the *H.polymorpha PEX10* coding region under transcriptional control of the *H.polymorpha* AOX promoter (P_{AOX}) (Tan *et al.*, 1995).

Generation of α-Pex14p antibodies

The Protein Fusion and Purification System (New England Biolabs, Beverly, MA) was used for the production of a maltose-binding protein (MBP)–Pex14p fusion protein in *E.coli*. A 1.3 kb *Xba*I (blunt-ended)–*Xho*II fragment encoding all but the first 37 amino acids of Pex14p was cloned in-frame behind the *malE* gene by insertion of the fragment into the *Xmn*I- and *Bam*HI-digested pMAL-c2 vector. Expression of the *malE–PEX14* chimeric gene under control of the P_{tac} promoter was induced by the addition of 0.3 mM isopropylthiogalactopyranoside. Purification of the MBP–Pex14p fusion protein using amylose resin and the separation of the Pex14p portion from MBP using Factor Xa protease were performed according to the instructions of the supplier. Purified Pex14p was used to immunize rabbits.

Construction of PEX14 overexpression strains

For overexpression, *PEX14* was cloned into pHIPX4-B and pHIPX5 behind the P_{AOX} and amine oxidase promoter (P_{AMO}), respectively. To construct pHIPX4-B, a 0.9 kb *Hin*dIII–*Nae*I (blunt-ended) fragment of the *PEX3* gene (Baerends *et al.*, 1996a) was subcloned into *Sma*I- and *Hin*dIII-digested pBluescriptIISK$^+$ (pBS). A 0.9 kb *Spe*I (blunt-ended)–*Sal*I fragment from the resulting plasmid was then subcloned into *Hin*dIII (blunt-ended)- and *Sal*I-digested pHIPX4, producing pHIPX4-B. A *Bam*HI site was introduced just 5′ of the *PEX14* initiation codon using PCR with the PEX14ATG primer (5′-GGGGGATCCATGTCTCAACA-GCCA-3′), the M13/pUC sequencing primer and a subclone carrying a 1.5 kb *Dra*I–*Eco*RV fragment of *PEX14* in the *Sma*I site of pBS (pBS1.5P14) as a template. From the amplified 1.3 kb *PEX14*-specific fragment, a 0.12 kb *Bam*HI–*Stu*I fragment was subcloned between the *Bam*HI and *Stu*I sites of pBS1.5P14; the resulting plasmid was named pBS1.3P14. From this plasmid, a 1.3 kb *Bam*HI–*Eco*RV fragment containing *PEX14* was inserted between the *Bam*HI and *Sma*I sites of pHIPX4-B and pHIPX5. These final overexpression plasmids were named pP_{AOX}PEX14 and pP_{AMO}PEX14, respectively.

Biochemical methods

Preparation of crude extracts and cell fractionations were performed as described (Douma *et al.*, 1985), except that 1 mM phenylmethylsulfonyl fluoride and 2.5 μg/ml of leupeptin were added. Peroxisomal peak fractions were subjected to carbonate extraction or high salt treatment (Baerends *et al.*, 1996). The 30 000 *g* pellet, obtained after differential centrifugation of homogenized protoplasts (Douma *et al.*, 1985) was used for flotation centrifugation as described by Goodman *et al.* (1986).

Protein concentrations were determined as described by Bradford (1976) using bovine serum albumin as standard. SDS–PAGE was carried out as described (Laemmli, 1970). Western blotting was performed using either the Protoblot immunoblotting system (Promega Biotec) or the ECL system (Amersham Corp., UK). The relative Pex14p levels were determined by laser densitometric scanning of Western blots, decorated with specific antibodies against Pex14p.

Electron microscopy

Cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously (Veenhuis and van der Klei, 1990). Immunolabelling was performed on ultrathin sections of Unicryl-embedded cells using specific antibodies against selected *H.polymorpha* peroxisomal proteins.

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