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 di-hydroxyacetone 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\(_2\)O\(_2\), 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 \(\beta\)-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 methylootropic yeast *Hansenula polymorpha*, peroxisomes are involved in the metabolism of several growth substrates and are maximally induced during methylothetic 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\(_3\)-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
The Hansenula polymorpha PEX14 gene

Fig. 1. Demonstration of peroxisomal remnants (arrow) in methanol-incubated cells of pex14-1 (A). In the complemented strain, a peroxisomal profile is evident (KMnO₄; 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 ~3×10⁴ 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 prototrophic 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 DraI–EcoRV subfragment that contained both ORF351 and ORF131 complemented the pex14-1 mutant while a 0.6 kb XhoI–EcoRV 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 DraI–EcoRV 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 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
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, AarII; Dr, DraI; Ec, EcoRI; EV, EcoRV; Ps, PstI; Sp, SphI; St, StuI; Xb, XbaI. (B) Correct integration of the Δpex14 fragment was demonstrated by Southern blot analysis. Chromosomal DNA (15 µg) isolated from wild-type (WT) and Δpex14 cells was digested with EcoRI, PstI and XbaI. The 1.1 kb XbaI–EcoRV 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 with the H. polymorpha URA3 gene (Figure 5A). After transformation of an H. polymorpha leu1.1 ural strain 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 (Δpex14) could not grow on methanol and lacked normal peroxisomes. The Δpex14 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 Δpex14 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 Δpex14 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 Δpex14 cells, incubated in batch cultures on methanol or grown in a carbon-limited chemostat on glucose/choline, revealed that the
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 Δpex14 strain, Pex10p, a homologous *H. polymorpha* peroxisomal membrane protein, was overproduced in the strain. For this purpose, a plasmid that carries *PEX10* under the control of the *H. polymorpha* AOX promoter was transformed into the Δpex14 strain. As shown before (Veenhuis et al., 1996), overproduction of Pex10p leads to the proliferation of the peroxisomal vesicles in Δpex strains. In Δpex14[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 Δpex14 strain, indeed represent peroxisomal membrane remnants.

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**Fig. 6.** Electron micrographs showing the morphology of Δpex14 cells. (A) The overall morphology of Δpex14 cells incubated in methanol-containing media. Peroxisomal membrane remnants are indicated by the arrow. The number of these vesicles is strongly enhanced in Δpex14 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 Δpex14 cells, these membranes are proliferated and specifically labelled in experiments using α-Pex10p antibodies (E). (For key, see Figure 1.)
**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.

**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[PaxPex14]) 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 Δpex14 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 methylene glycol, 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 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...
The Hansenula polymorpha 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 Δpex14[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 Δpex14 strain. In this way, the levels of Pex14p could be varied by manipulation of the growth conditions.

Cells of Δpex14[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 Δpex14[P_AMO PEX14] on methanol/methylamine were largely comparable with those of wild-type cells. Electron microscopical analyses of samples, taken
Western blot analysis of crude extracts from Δpex14 cells containing Pₐₒₓ Pₐₓ14. 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.

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[Pₐₒₓ Pₐₓ14] 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 conventional method for peroxisome purification) 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 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 Δpex14 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

<table>
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<th>Strains/plasmids</th>
<th>Relevant properties</th>
<th>Source or reference</th>
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<tr>
<td>H. polymorpha</td>
<td>leu1::URA3 pex1-1 (originally named per10-108)</td>
<td>Titorenko et al. (1993)</td>
</tr>
<tr>
<td>pex14::::URA3</td>
<td>leu1::URA3 pex14::::URA3</td>
<td>CBS collection, The Netherlands</td>
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<td>wild-type</td>
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<tr>
<td>NCYC 495</td>
<td>leu1::URA3</td>
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<td>XL1-Blue MRF</td>
<td>Stratagene</td>
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<td>DH5α</td>
<td>supE44ΔlacU169(q80lacZΔM15)hisD17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Sambrook et al. (1989)</td>
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**Plasmids**

| pYT3 | Ap<sup>+</sup>, LEU2, HARS1 | Tan et al. (1995) |
| pHIPX4-B | Km<sup>+</sup>, LEU2, P<sub>AOX</sub> | this study |
| pHIPX5 | Km<sup>+</sup>, LEU2, P<sub>AMO</sub> | this study |
| pBluescript II SK<sup>+</sup> | Ap<sup>+</sup> | Stratagene |
| pMAL-c2 | Ap<sup>+</sup>, P<sub>mal</sub>malE | New England Biolabs |
| pET4 | Km<sup>+</sup>, LEU2, P<sub>AOX</sub>P<sub>EX10</sub> | Tan et al. (1995) |
| pP<sub>EX14</sub>-3 | Ap<sup>+</sup>, LEU2, HARS1, 4.5 kb-P<sub>EX14</sub> | this study |
| pBS1.2P14 | Ap<sup>+</sup>, 3.2 kb-P<sub>EX14</sub> | this study |
| pBS1.5P14 | Ap<sup>+</sup>, 1.5 kb-P<sub>EX14</sub> | this study |
| pBS1.3P14 | Ap<sup>+</sup>, 1.3 kb-P<sub>EX14</sub> | this study |
| pP<sub>AOX</sub>P<sub>EX14</sub> | Km<sup>+</sup>, LEU2, P<sub>AOX</sub>P<sub>EX14</sub> | this study |
| pP<sub>AMO</sub>P<sub>EX14</sub> | Km<sup>+</sup>, LEU2, P<sub>AMO</sub>P<sub>EX14</sub> | 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* Δpex14 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 immunochemical 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<sup>–</sup> again (i.e. Mut<sup>–</sup> and absence of peroxisomes); a major difference between the peroxisomal remnants in *Apex14* 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 medium containing 0.67% (w/v) yeast nitrogen base supplemented with 1% (w/v) glucose (YNB) 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.

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Cloning and sequence analysis of the PEX14 gene

To isolate the PEX14 gene, the Hansenula polymorpha pex4-1 mutant (Titorenko et al., 1993) was transformed with the plasmids and ampicillin-resistant colonies were selected. The plasmids were used to transform E. coli and the resulting colonies were screened for the presence of the PEX14 gene. The sequence of the PEX14 gene was determined using the TBLASTN algorithm (Altschul et al., 1990) and the resulting sequence was used to design primers for PCR amplification.

Construction of a PEX14 deletion mutant

The disruption of the PEX14 gene was performed by homologous recombination. The PEX14 gene was amplified by PCR and used to transform E. coli DH5α, and the resulting recombinant plasmids were used to transform Hansenula polymorpha. The resulting transformants were selected on plates containing kanamycin and the presence of the PEX14 gene was confirmed by Southern blot analysis.

Generation of α-Pex14p antibodies

The α-Pex14p antibodies were generated by immunizing rabbits with the purified PEX14 protein. The antibodies were used to detect the Pex14p protein in western blots and immunofluorescence microscopy.

Biochemical methods

Preparation of crude extracts and cell fractionation were performed as described by Gnos et al. (1995). Peroxisomal membrane proteins were isolated by differential centrifugation and the protein content was measured using the Bradford assay.

Electron microscopy

Cells were fixed and embedded in resin, and thin sections were stained with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope.

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Electron microscopy

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

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