

# The ubiquitin-conjugating enzyme Pex4p of *Hansenula polymorpha* is required for efficient functioning of the PTS1 import machinery

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We have cloned the *Hansenula polymorpha* *PEX4* gene by functional complementation of a peroxisome-deficient mutant. The *PEX4* translation product, Pex4p, is a member of the ubiquitin-conjugating enzyme family. In *H. polymorpha*, Pex4p is a constitutive, low abundance protein. Both the original mutant and the *pex4* deletion strain ( $\Delta pex4$ ) showed a specific defect in import of peroxisomal matrix proteins containing a C-terminal targeting signal (PTS1) and of malate synthase, whose targeting signal is not yet known. Import of the PTS2 protein amine oxidase and the insertion of the peroxisomal membrane proteins Pex3p and Pex14p was not disturbed in  $\Delta pex4$  cells. The PTS1 protein import defect in  $\Delta pex4$  cells could be suppressed by overproduction of the PTS1 receptor, Pex5p, in a dose-response related manner. In such cells, Pex5p is localized in the cytosol and in peroxisomes. The peroxisome-bound Pex5p specifically accumulated at the inner surface of the peroxisomal membrane and thus differed from Pex5p in wild-type peroxisomes, which is localized throughout the matrix. We hypothesize that in *H. polymorpha* Pex4p plays an essential role for normal functioning of Pex5p, possibly in mediating recycling of Pex5p from the peroxisome to the cytosol.

**Keywords:** peroxisome-deficient mutant/protein import/PTS1 receptor/yeast

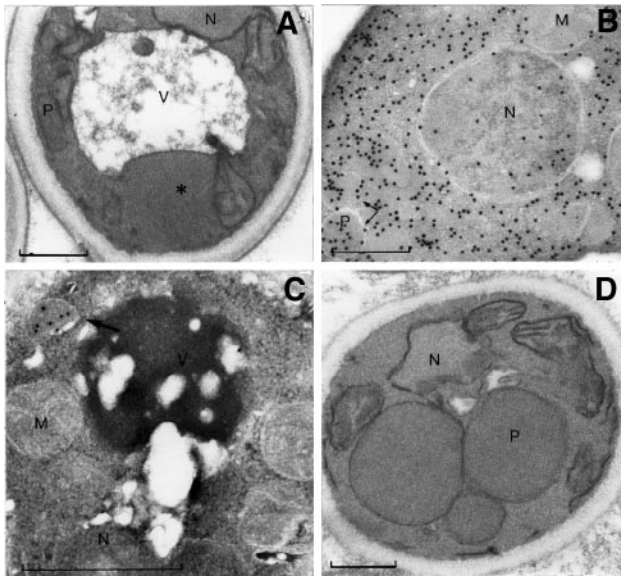
## Introduction

A characteristic feature of eukaryotic cells is that specific cellular functions are compartmentalized. Of these compartments, peroxisomes are the most versatile because they are involved in a variety of metabolic conversions which are dependent on the cell type and organism in which they occur (van den Bosch *et al.*, 1992). In yeasts, peroxisomes are involved in the metabolism of specific carbon and/or nitrogen sources, e.g. methanol, D-alanine, primary amines or oleic acid (van der Klei and Veenhuis, 1996). In these organisms, the metabolic significance and

protein contents of the organelles are therefore largely prescribed by the prevailing growth conditions of the cells.

Peroxisomes do not contain DNA and also lack a protein-synthesizing machinery. Hence, all peroxisomal protein components are recruited from the cytosol (reviewed by McNew and Goodman, 1996). Peroxisomal proteins are generally thought to be synthesized on free cytosolic polysomes, with the probable exception of some membrane proteins (Bodnar and Rachubinski, 1991). For import of matrix proteins, two peroxisomal targeting signals (PTSs) have been identified: PTS1, located at the extreme C-terminus, consists of the sequence SKL-COOH or conserved variants of this tripeptide (Gould *et al.*, 1990) and is found in most matrix proteins including the key peroxisomal enzymes of methanol metabolism in *Hansenula polymorpha*, alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT). PTS2 (RL/I-X<sub>5</sub>-H/QL), located near the N-terminus (de Hoop and AB, 1992), is found in only few yeast proteins [e.g. *H. polymorpha* amine oxidase (AMO)]. A few matrix proteins are known which contain neither a PTS1 nor a PTS2. An example of such a protein is *H. polymorpha* peroxisomal malate synthase (MS). Interestingly, import of this protein is also dependent on the PTS1 receptor, Pex5p (van der Klei *et al.*, 1995). At present, only limited information is available on targeting and insertion mechanisms of peroxisomal membrane proteins. Goodman and co-workers identified a region in *Candida boidinii* PMP47 that is able to target the protein to the peroxisomal membrane (Dyer *et al.*, 1996). Unexpectedly, some peroxisomal membrane proteins contain regions which can target reporter proteins to the endoplasmic reticulum (ER) (Baerends *et al.*, 1996).

Several genes essential for peroxisomal protein import have been cloned by functional complementation of yeast *pex* mutants (for reviews see Subramani 1993, 1996). Among these are the two genes encoding the PTS1 (*PEX5*) and PTS2 (*PEX7*) receptor proteins and two putative docking proteins which bind the PTS receptors (reviewed by Erdman *et al.*, 1997). The exact subcellular location of Pex5p and Pex7p is still controversial to some extent, even within one organism (reviewed by Rachubinski and Subramani, 1995; Subramani, 1996). In wild-type cells of the methylotrophic yeast *H. polymorpha*, the PTS1 receptor (HpPex5p) has a dual location, namely inside peroxisomes and in the cytosol (van der Klei *et al.*, 1995). Based on these locations, we proposed that *H. polymorpha* Pex5p may shuttle PTS1 proteins from the cytosol into the peroxisomal matrix. After dissociation of the receptor and its cargo molecule in the peroxisomal matrix, the receptor recycles back to the cytosol for transport of additional cytosolically synthesized matrix proteins (van der Klei and Veenhuis, 1996). However, definite experimental proof for this hypothesis is still lacking.



**Fig. 1.** (A) Morphology of a methanol-induced cell of the original *H. polymorpha* mutant *per2-1*, showing a small elongated peroxisome in conjunction with a cytosolic AO crystalloid (\*). The structure of the crystalloid is poorly preserved due to the  $\text{KMnO}_4$  fixation procedure. (B) Immunocytochemically, the peroxisomes in these cells contain AO protein (arrows);  $\alpha$ -AO-specific labelling is also observed in the cytosol, including the nucleus. (C) In ethanol/ethylamine-grown *per2-1* cells, AMO is localized exclusively in the peroxisomal matrix. (D) The presence of normal peroxisomes in the functionally complemented *per2-1* mutant. The electron micrographs in (A) and (D) were of  $\text{KMnO}_4$ -fixed cells, and those in (B) and (C) were of cells fixed in glutaraldehyde ( $\alpha$ -AMO/GAR-gold). M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 0.5  $\mu\text{m}$ .

Here, we describe the *H. polymorpha* *PEX4* gene and its protein product, Pex4p, which belongs to the family of ubiquitin-conjugating (UBC) enzymes. We show that Pex4p is required for efficient functioning of the PTS1 import machinery, possibly by mediating Pex5p recycling.

## Results

### Characterization of the *per2-1* mutant and cloning of the corresponding gene

The *H. polymorpha per2-1* mutant (Titorenko *et al.*, 1993) was isolated within a collection of mutants which were unable to grow on methanol ( $\text{Mut}^-$  phenotype). In methanol-induced *per2-1* cells, a few small peroxisomes were present, together with a large cytosolic crystalloid, which is indicative of peroxisome deficiency in *H. polymorpha* (Figure 1A). Immunocytochemistry revealed that the small peroxisomes contained the typical PTS1 proteins AO and DHAS, while the bulk of these proteins resided in the cytosol (Figure 1B, AO; DHAS not shown). Also, CAT was found predominantly in the cytosol (not shown), whereas the PTS2 protein AMO was located exclusively in peroxisomes (Figure 1C). These results indicated that in *per2-1* cells, specifically the import of PTS1 proteins may be impaired.

The corresponding gene was cloned by functional complementation of the *per2-1 leu1.1* mutant using an *H. polymorpha* genomic DNA library. A plasmid, designated pHRP2-*PER2*, was shown to be able to complement the  $\text{Mut}^-$  phenotype and to restore normal peroxisome assembly in the *per2-1* mutant (Figure 1D). Subsequent

HpPex4p	MSS-----TEKRLLEKYRAVKKELTEKRSF--IHDGTIVDL	34
PpPex4p	MSA-----EKRLLEQYRSILKEQKQSGASTLSSNGILDL	35
ScPex4p	MPNFWILENRRSYTSDTCMSRIVKEYKVLKTLASDDPIANPYRGIIESL	50
	* * * * *	
HpPex4p	HPL-EDGLFRWSAVIRGPDQSPFEDALWKEIDIPTNYPLDPPKIKFVVF	83
PpPex4p	KPVSEDNFYKWTAKLKGPTDTGYQDAFWELQIDIRSNYPTQPKFTFVS	85
ScPex4p	NPIDETDLSEKWEAIISGSDTPYENHQFRLLLEVSPSYPMNPKEISFM--	98
	* * * * *	
HpPex4p	GEEKIRQLQRKTS-----SGARKV---CYKMPHPNVNFKTGEICLD	121
PpPex4p	DDIPNRRQRQTNIQDDDEFEGAEKEVLRHCYRMPHPNIAFNTGEICLD	135
ScPex4p	-----QNNIL-HC-----NVKSATGEICLN	117
	* * * * *	
HpPex4p	ILQOK-WSPAWTLSALVAIVVLLANPEPLSPNLIDMANLLKCDTAYK	170
PpPex4p	ILQAK-WTPAWTLSSALTAIVLLNDPEPLSPDLIDMANLMKINDLKAYN	184
ScPex4p	ILKPEEWTFFVMDLLHCHAVHVRPFCVCSPLDVGIGNIRCGDMASYQ	167
	* * * * *	
HpPex4p	DLVHYIYIAKYSAYESNDV--	188
PpPex4p	SLIEYVGRYSIEEEVYILN	204
ScPex4p	GIVKYFLAERERINN---H	183
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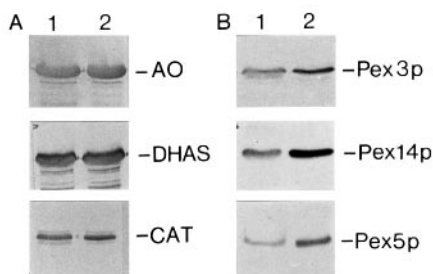
**Fig. 2.** Alignment of the deduced amino acid sequences of *H. polymorpha* *PEX4* (HpPex4p), *P. pastoris* *PEX4* (PpPex4p; Crane *et al.*, 1994) and *S. cerevisiae* *PEX4* (ScPex4p; Wiebel and Kunau, 1992). The conserved cysteine residue in the active site is in bold italic typeface. Identical amino acids are indicated by an asterisk, similar ones by a dot.

subcloning revealed that the complementing activity in pHRP2-*PER2* was contained within a 2.0 kb *EcoRI*-*NheI* subfragment. The DNA sequence of this fragment displayed one open reading frame (ORF) of 564 bp, encoding a protein of 188 amino acids with a calculated mol. wt of 21.5 kDa (accession No. AF061604). A search of databases revealed sequence similarity of this protein to members of the UBC protein family, including Pex4p from *Pichia pastoris* (53% identity; formerly Pas4p; Crane *et al.*, 1994) and Pex4p from *Saccharomyces cerevisiae* (32% identity; formerly Pas2p; Wiebel and Kunau, 1992). This implies that the *PER2* gene in fact represents the *H. polymorpha* *PEX4* orthologue. An alignment of the known *PEX4* protein products revealed that the C-terminal half, including the conserved active site cysteine involved in ubiquitin binding, is highly conserved (Figure 2). The deduced amino acid sequence of *H. polymorpha* Pex4p (HpPex4p) does not contain any of the known peroxisomal targeting signals and has no predicted membrane-spanning regions.

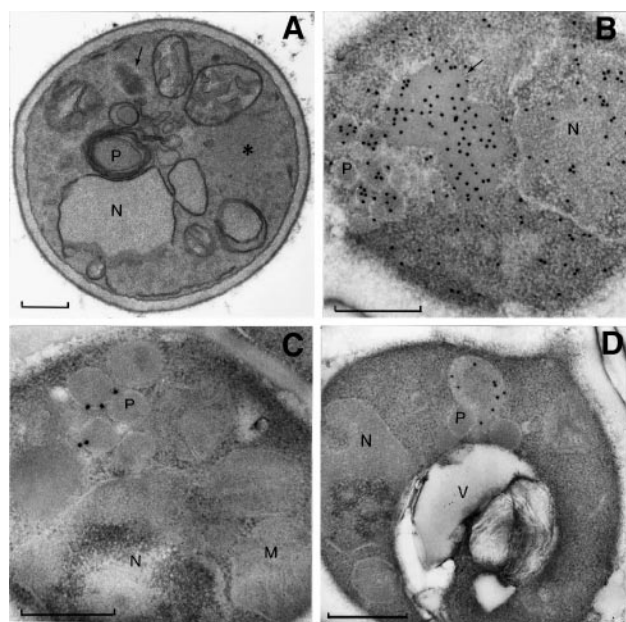
### Characterization of a *pex4* deletion strain

To obtain further insight into the function of HpPex4p, we constructed an *H. polymorpha pex4* deletion strain ( $\Delta pex4$ ) by replacing most of the *PEX4* ORF, including the translation initiation site, by a DNA fragment containing the *H. polymorpha URA3* gene. Southern blot analysis of a selected  $\text{Mut}^- \text{Ura}^+$  transformant revealed that the fragment was integrated correctly in the genome (data not shown). After transformation of  $\Delta pex4$  with the *per2-1* complementing plasmid pHRP2-*PER2*, growth of the transformants on methanol was restored, confirming that the correct gene had been disrupted. The  $\Delta pex4$  strain subsequently was mated with the original *per2-1* mutant. The resulting diploids all were  $\text{Mut}^-$ ; after sporulation, no  $\text{Mut}^+$  cells were observed (>300 spore products tested), demonstrating that *per2-1* and  $\Delta pex4$  are closely linked and most likely are alleles of the same gene.

Western blot analysis (Figure 3) revealed that various peroxisomal membrane and matrix proteins were synthesized normally in  $\Delta pex4$  after incubation of cells for 24 h



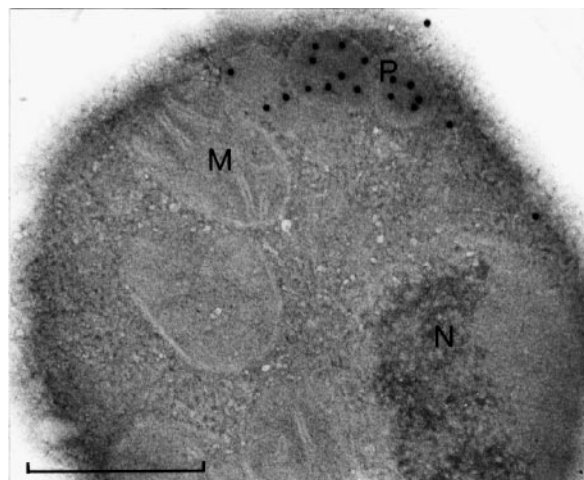
**Fig. 3.** Protein levels of peroxisomal enzymes and peroxins in wild-type *H. polymorpha* and the  $\Delta pex4$  deletion mutant. Western blots were prepared from crude extracts of methanol-induced wild-type (lanes 1) or  $\Delta pex4$  cells (lanes 2). Equal amounts of protein were loaded per lane. The blots were decorated using specific polyclonal antibodies as indicated.



**Fig. 4.** (A) Morphology (arrow = aggregate; \* = crystalloid) and (B–D) immunocytochemical demonstration of various proteins in  $\Delta pex4$ . (B) AO protein (arrow = AO aggregate); (C) Pex14p; (D) Pex3p. The structure of the crystalloid in (A) is poorly preserved due to the  $KMnO_4$  fixation procedure. The electron micrograph in (A) is of a  $KMnO_4$ -fixed cell, those in (B–D) are of cells fixed in glutaraldehyde (GAR–gold and specific antisera against the indicated proteins). M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 0.5  $\mu m$ .

in methanol-containing media, conditions which induce strong peroxisome proliferation in wild-type cells. As shown in Figure 3A, the major peroxisomal matrix enzymes (AO, CAT and DHAS) were present at wild-type levels (Figure 3A). The peroxins Pex3p, Pex5p and Pex14p were found at enhanced levels in  $\Delta pex4$  cells compared with wild-type controls (Figure 3B).

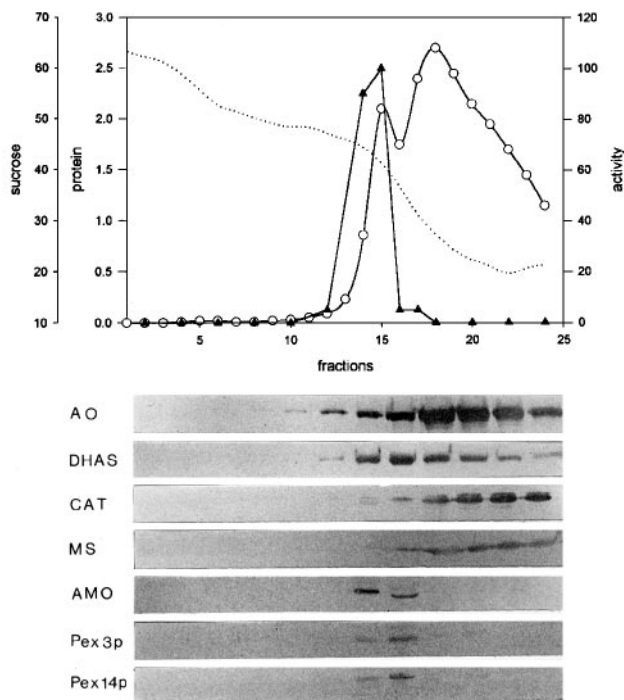
Electron microscopy revealed that the morphological phenotype of  $\Delta pex4$  cells was indistinguishable from that of the original mutant *per2-1*. Methanol-induced cells contained few relatively small peroxisomes which occasionally were associated with membranous layers, in conjunction with a cytosolic crystalloid and aggregates (Figure 4A). Both the aggregates and the crystalloid contained AO and DHAS protein, but not CAT and MS, as determined by immunocytochemistry (not shown, except



**Fig. 5.** Immunocytochemical demonstration of AMO protein inside peroxisomes of  $\Delta pex4$  cells grown on ethanol/ethylamine. The cells were fixed in glutaraldehyde (GAR–gold and  $\alpha$ -AMO). M, mitochondrion; N, nucleus; P, peroxisome. Bar = 0.5  $\mu m$ .

for AO in Figure 4B). As in *per2-1* cells, the labelling patterns after immunocytochemistry indicated that a major fraction of the PTS1 matrix enzymes AO, DHAS and CAT was mislocated to the cytosol (Figure 4B, AO; DHAS and CAT not shown). Also MS, which lacks any known targeting signal, was cytosolic (not shown). Using antibodies against the peroxisomal membrane proteins Pex3p and Pex14p, labelling was confined to the peroxisomal membrane and the peroxisome-associated membranous layers (Figure 4C and D). The PTS2 protein AMO, using  $\alpha$ -AMO on ultrathin sections of ethanol/ethylamine-grown cells, was found solely in the peroxisomal matrix (Figure 5).

The immunocytochemical results were confirmed biochemically. A post-nuclear supernatant (PNS) was prepared from  $\Delta pex4$  cells grown on a mixture of glycerol and methanol in the presence of methylamine as sole nitrogen source to induce AMO protein and subsequently subjected to sucrose gradient centrifugation. The protein profile (Figure 6) revealed that in this gradient the typical peroxisomal protein peak was lacking, which is normally found at a density corresponding to 53–56% sucrose (fraction 6) in gradients prepared from wild-type cells. AO, DHAS, CAT and MS were now found predominantly in the upper part of the gradient, indicative of a cytosolic location of these proteins (fractions 18–24). The relative difference in location between AO and DHAS, compared with CAT and MS, most likely reflects the presence of AO- and DHAS-containing aggregates. AMO was found exclusively in fractions 14–16 and fully co-fractionated with the peroxisomal membrane proteins Pex3p and Pex14p. Hence, fractions 14–16 most likely contain the small peroxisomes present in  $\Delta pex4$  cells. These fractions contained only a portion of AO, DHAS, CAT and MS protein, which confirms that there is a partial block in the import of these proteins. Taken together, these data indicate that disruption of the *PEX4* gene resulted in a decreased efficiency in the import of matrix proteins containing a PTS1 and of MS. PTS2 protein import and the insertion of peroxisomal membrane proteins is unaffected in  $\Delta pex4$  cells.

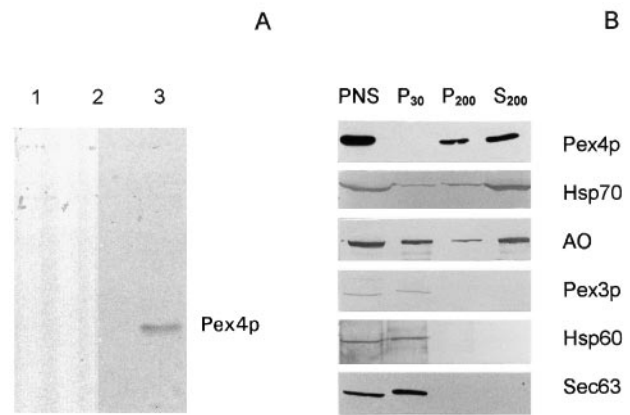


**Fig. 6.** Sucrose density gradient prepared from a post-nuclear supernatant of glycerol/methanol/methylamine-grown  $\Delta pex4$  cells. The dotted line shows the sucrose concentration as a percentage (w/w); the protein concentration patterns ( $\circ$ ; mg/ml) and the distribution of the mitochondrial marker protein cytochrome c oxidase ( $\blacktriangle$ ; expressed as a percentage of the peak activity, which was arbitrarily set at 100) are shown. The Western blots show the distribution of AO, DHAS, CAT, MS, AMO, Pex3p and Pex14p. Equal portions of the even fractions (2–24) were loaded in each lane.

#### Pex4p is a low abundance, constitutive protein

Polyclonal antibodies were raised against HpPex4p. Western blot experiments using these antibodies and crude extracts prepared from wild-type *H. polymorpha* invariably failed to identify a cross-reacting protein band, independently of the cultivation conditions used (Figure 7A). In order to test whether this result was due to very low *PEX4* expression levels in wild-type cells, HpPex4p-overproducing  $\Delta pex4$  strains were constructed, containing one or multiple copies of *PEX4* under control of the strong AO promoter ( $P_{AOX}$ ). On Western blots of methanol-induced  $\Delta pex4::P_{AOX}PEX4^{1\times}$  cells, the  $\alpha$ -HpPex4p antiserum recognized a single protein band with an apparent mol. wt. of ~22 kDa, which is in good agreement with the calculated molecular weight of the *PEX4* gene product (21.5 kDa). Since a clear-cut dose-response relationship existed between the intensity of the cross-reacting band and the number of copies of  $P_{AOX}PEX4$  present in the transformants (data not shown), we concluded that the antiserum specifically recognized HpPex4p.

To obtain additional evidence that in wild-type *H. polymorpha* the *PEX4* expression levels are very low, the *PEX4* promoter activity was studied using  $\beta$ -lactamase as a reporter protein (Waterham *et al.*, 1994). Enzyme activity measurements revealed that in crude extracts prepared from methanol-grown cells of a strain, which contained a single copy of the  $\beta$ -lactamase gene under control of the *PEX4* promoter, very low  $\beta$ -lactamase activities were present (Table I). Comparable activities were found in glucose- and methanol-grown cells, suggesting that *PEX4*



**Fig. 7.** (A) Western blot showing the specificity of the  $\alpha$ -Pex4p antiserum. In crude extracts prepared from methanol-induced  $\Delta pex4$  cells (lane 1) or methanol-grown wild-type cells (lane 2), a cross-reacting band was not detectable. In cells overproducing HpPex4p ( $\Delta pex4::P_{AOX}PEX4^{1\times}$ ), a protein band with the apparent mol. wt. of 22 kDa was evident (lane 3). (B) Subcellular location of HpPex4p in methanol-grown  $\Delta pex4::P_{AOX}PEX4^{2\times}$  cells. A post-nuclear supernatant (PNS) was subjected to differential centrifugation. Western blots were prepared from the different fractions ( $P_{30}$ , 30 000 g pellet;  $P_{200}$ , 200 000 g pellet;  $S_{200}$ , 200 000 g supernatant) and decorated with antibodies against HpPex4p and different marker proteins (Hsp70, cytosol; AO, peroxisomal matrix; Pex3p, peroxisomal membrane; hsp60, mitochondrial matrix; Sec63, endoplasmic reticulum). The presence of a portion of the peroxisomal matrix protein AO in  $S_{200}$  is due to partial leakage of matrix protein from the fragile peroxisomes (see also Figure 9). Equal portions were loaded per lane.

**Table I.** Promoter activity of  $P_{PEX4}$

Strain	Growth substrate	
	Glucose	Methanol
Wild-type:: $P_{PEX4}\beta$ -lactamase	0.1	0.1
Wild-type:: $P_{PEX8}\beta$ -lactamase	0.1	0.3
Wild-type:: $P_{AOX}\beta$ -lactamase	0.0	10.5
Wild-type	0.0	0.0

$\beta$ -Lactamase activities in crude extracts prepared from glucose- or methanol-grown cells, harvested at the late exponential growth phase. The activities are expressed as  $\Delta E_{486}/\text{min}/\text{mg}$  protein.

is constitutively expressed. The  $\beta$ -lactamase activity amounted to ~1% of the values obtained in control experiments in which the  $P_{AOX}$  was used (Table I). Also, relative to  $P_{PEX8}$  (Waterham *et al.*, 1994),  $P_{PEX4}$ -driven  $\beta$ -lactamase synthesis is low. Since Pex8p is a low abundance protein in *H. polymorpha* wild-type (Waterham *et al.*, 1994), these data confirm that HpPex4p is present at low levels in *H. polymorpha*.

HpPex4p was also not detected in the various fractions obtained after fractionation of cell homogenates of *H. polymorpha* wild-type cells. Moreover, immunocytochemistry was inconclusive due to too low labelling intensities. We therefore studied the location of HpPex4p in a *PEX4*-overexpressing strain. Physiological experiments indicated that cells of  $\Delta pex4$  strains containing one, two or four copies of the  $P_{AOX}PEX4$  overexpression cassette grew normally on methanol, independently of the amount of HpPex4p produced by the cells (data not shown). Electron microscopy revealed that in  $\Delta pex4::P_{AOX}PEX4^{4\times}$  cells the number and volume fraction of peroxisomes was not

significantly altered compared with wild-type controls. Hence, *PEX4* overexpression did not affect peroxisome assembly and function in *H. polymorpha*.

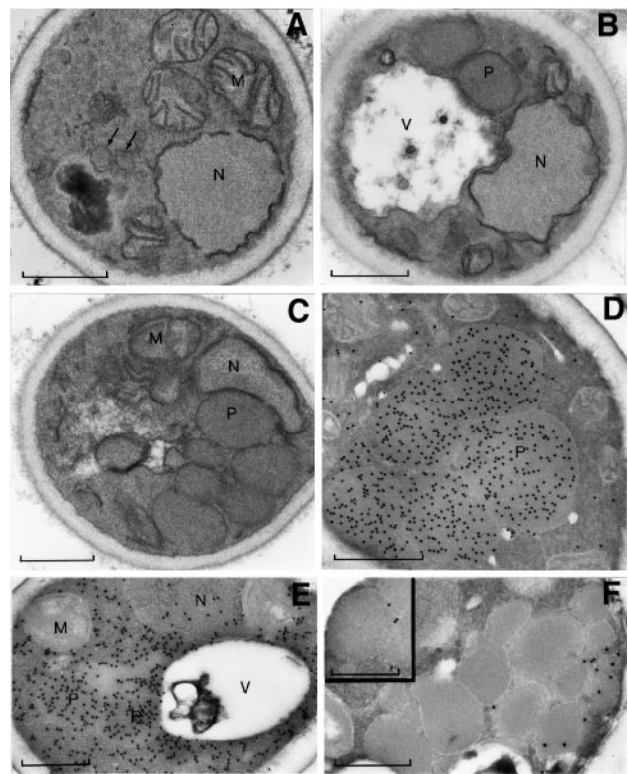
Upon differential centrifugation of methanol-induced  $\Delta pex4::P_{AOX}PEX4^{2\times}$  cells, HpPex4p was found predominantly in the 200 000 g supernatant, whereas a portion of the protein was recovered in the 200 000 g pellet. As shown in Figure 7B, HpPex4p was undetectable in the 30 000 g pellet in which peroxisomal, mitochondrial and ER marker proteins predominantly sedimented. We therefore conclude that in this strain, HpPex4p is not bound to these organelles. However, the pelletable portion may represent Pex4p that is bound to small membrane structures or large protein complexes. Upon sucrose centrifugation of homogenized  $\Delta pex4::P_{AOX}PEX4^{2\times}$  cells, HpPex4p remained in the upper fractions of the gradient, indicative of a cytosolic location. HpPex4p was invariably absent in peroxisomal peak fractions (data not shown).

#### Overproduction of HpPex5p suppresses the PTS1 protein import defect in $\Delta pex4$ cells

In  $\Delta pex4$  cells, the import of PTS1 proteins is selectively affected, whereas the PTS2 protein AMO is imported normally. In  $\Delta pex5$  cells, thus lacking the PTS1 receptor Pex5p, the import of the same subset of matrix proteins (AO, DHAS, CAT and MS) is fully blocked (van der Klei *et al.*, 1995). We therefore reasoned that Pex4p might be important for proper functioning of Pex5p. One possibility is that Pex4p is important for recycling of Pex5p from peroxisomes back to the cytosol (Dodt and Gould, 1996; van der Klei and Veenhuis, 1996). This could explain the observed reduction in Pex5p-dependent import in  $\Delta pex4$  cells since individual Pex5p molecules may guide only one or a few proteins to the organelle, instead of mediating multiple rounds of import. In line with this reasoning, the import defect should at least be partly compensated by making more PTS1 receptor molecules available. In order to test this hypothesis, we overexpressed the *PEX5* gene under control of  $P_{AOX}$  in  $\Delta pex4$  cells ( $\Delta pex4::P_{AOX}PEX5$ ). Strains were selected that contained one, two or multiple (most likely four) copies of  $P_{AOX}PEX5$ . Western blots, prepared from methanol-induced cells of the transformants, showed that HpPex5p was indeed overproduced in these strains. As expected, the highest amounts of HpPex5p were observed in crude extracts of  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells (~100 times wild-type levels; data not shown).

Electron microscopic analysis of ultrathin sections of  $KMnO_4$ -fixed cells of the various transformants revealed that overproduction of HpPex5p in  $\Delta pex4$  strains led to a significant increase in both the size and the number of peroxisomes. Typical examples of each of the transformants are shown in Figure 8A–C. A clear-cut dose-response relationship was observed between the restoration of peroxisome development and the amount of HpPex5p synthesized (Table II).

Immunocytochemically, using  $\alpha$ -AO antibodies and ultrathin sections of each of the  $\Delta pex4::P_{AOX}PEX5^{n\times}$  strains revealed significant cytosolic labelling, indicating that a portion of this protein is still located in the cytosol. The cytosolic labelling intensity decreased with increasing amounts of HpPex5p in the cells, suggesting that the amount of AO incorporated into peroxisomes was proportional to the rate of overproduction of HpPex5p (Figure



**Fig. 8.** Overproduction of Pex5p suppresses the PTS1 import defect in  $\Delta pex4$  cells. (A) Methanol-induced  $\Delta pex4$  cells contain only a few small peroxisomes. (B) In identically grown  $\Delta pex4::P_{AOX}PEX5^{1\times}$  cells, the size of the peroxisomes is increased. (C)  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells contain several large peroxisomes, which resemble organelles in methanol-grown wild-type cells. (D) Using  $\alpha$ -AO antisera, the cytosolic labelling is very low in  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells. (E) In  $\Delta pex4::P_{AOX}PEX5^{2\times}$  cells, an intermediate situation is observed. (F) Typical labelling of small developing peroxisomes using antibodies against Pex10p in  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells, which is comparable with the wild-type situation (Tan *et al.*, 1995). Inset:  $\alpha$ -Pex3p. The electron micrographs in A–C are of  $KMnO_4$ -fixed cells; those in (D–F) were fixed in glutaraldehyde (GAR–gold, uranylacetate and antibodies against the indicated proteins). M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 0.5  $\mu$ m.

**Table II.** Number and volume fraction of peroxisomes in various *H. polymorpha* strains

Strain	Peroxisome number	Volume fraction
$\Delta pex4$	0.1	0.15
$\Delta pex4::P_{AOX}PEX5^{1\times}$	0.3	4.3
$\Delta pex4::P_{AOX}PEX5^{2\times}$	0.8	16.9
$\Delta pex4::P_{AOX}PEX5^{4\times}$	1.2	23.4
Wild-type	1.1	24.9

Peroxisome number and volume fraction in cells incubated for 24 h in methanol-containing media. The number of peroxisomes is expressed as the average number per section. The volume fraction of peroxisomes is given as a percentage of the cytoplasm.

8D and E). Similar results were obtained when antibodies against DHAS were used in immunocytochemical experiments (data not shown). The membrane proteins Pex3p, Pex10p and Pex14p were found exclusively at the peroxisomal membrane of these cells (Pex14p not shown). As in wild-type *H. polymorpha* (Tan *et al.*, 1995), specific Pex10p labelling was observed predominantly on the smaller organelles (Figure 8F).

The electron microscopic data were confirmed by cell fractionation studies performed on methanol-induced  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells, using identically grown  $\Delta pex4$  and wild-type cells as controls. As shown in Figure 9, a distinct protein and AO activity peak is observed after sucrose density centrifugation of homogenized  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells at 54% sucrose (fraction 6), which is lacking in the gradients of  $\Delta pex4$  cells but is evident in gradients prepared from wild-type cells. This indicates that in  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells, normal peroxisomes had developed. This was confirmed by the finding that Pex3p, which is an integral component of the peroxisomal membrane, also sedimented exclusively in fraction 6 in gradients of wild-type and  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells, in contrast to its location in fraction 18 in the gradient of  $\Delta pex4$  cells.

**Peroxisomal Pex5p is located at the inner surface of the peroxisomal membrane in  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells**

In immunocytochemical experiments, using specific  $\alpha$ -HpPex5p antibodies and ultrathin sections of methanol-induced  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells, labelling was located predominantly on the peroxisomal membrane (Figure 10B and C); in addition, labelling was observed randomly distributed over the cytosol. This labelling pattern was different from that obtained using wild-type cells, in which HpPex5p was found both throughout the peroxisomal matrix and in the cytosol (Figure 10A). Also, when HpPex5p was overproduced in wild-type cells, the protein was present both throughout the matrix and in the cytosol (not shown). Hence, the association of HpPex5p with the peroxisomal membrane in  $\Delta pex4::P_{AOX}PEX5^{4\times}$  apparently reflects the absence of HpPex4p in this strain.

In order to test whether the peroxisome-bound HpPex5p was present inside the peroxisomal matrix or bound to the outer surface of the organelle, a protease protection assay was performed. The results, summarized in Figure 11, show that degradation of HpPex5p in organellar fractions from methanol-induced  $\Delta pex4::P_{AOX}PEX5^{4\times}$  only occurred in the presence of Triton X-100. At the relatively low proteinase K (PK) concentration used (0.1 mg/ml), Pex5p was fully degraded in the presence of Triton X-100, whereas in the absence of the detergent, Pex5p was fully protected. A similar result was found for HpPex5p in organellar fractions derived from wild-type [ $P_{AOX}PEX5$ ] cells. Control blots revealed that the matrix protein catalase was fully protected against PK in the absence of Triton X-100, which indicates that the peroxisomes were intact. In the presence of Triton X-100, the low PK concentration did not allow complete degradation of CAT, but resulted in the formation of a degradation product. Degradation of Pex14p, a peripheral membrane protein exposed to the cytosol (Komori *et al.*, 1997), occurred to a similar extent both in the absence and presence of Triton X-100. Taken together, these results indicate that in the organellar pellets of both wild-type [ $P_{AOX}PEX5$ ] and  $\Delta pex4::P_{AOX}PEX5$ , HpPex5p is present in the organellar matrix (like CAT) and not at the outside of the organelle, exposed to the cytosol (like Pex14p). In addition, this experiment illustrated that Pex5p is very sensitive to PK in comparison with CAT and Pex14p.

**The PTS1 import defect in  $\Delta pex4$  is only suppressed by overexpression of PEX5**

Overexpression of other *H. polymorpha* PEX genes, e.g. PEX1, PEX3, PEX8 or PEX14, did not result in restoration of the peroxisomal protein import defect in  $\Delta pex4$  cells (Figure 10D,  $\Delta pex4::P_{AOX}PEX8$ ; others not shown). Hence, the peroxisomal protein import defect in  $\Delta pex4$  could not be suppressed simply by overproduction of any peroxin but was restored specifically by HpPex5p overproduction. However, the difference in location of Pex5p in wild-type and  $\Delta pex4$  cells (compare Figure 10A and C) clearly suggests that overexpression of Pex5p in  $\Delta pex4$  does not rescue the mechanistic defect in matrix protein import in these cells.

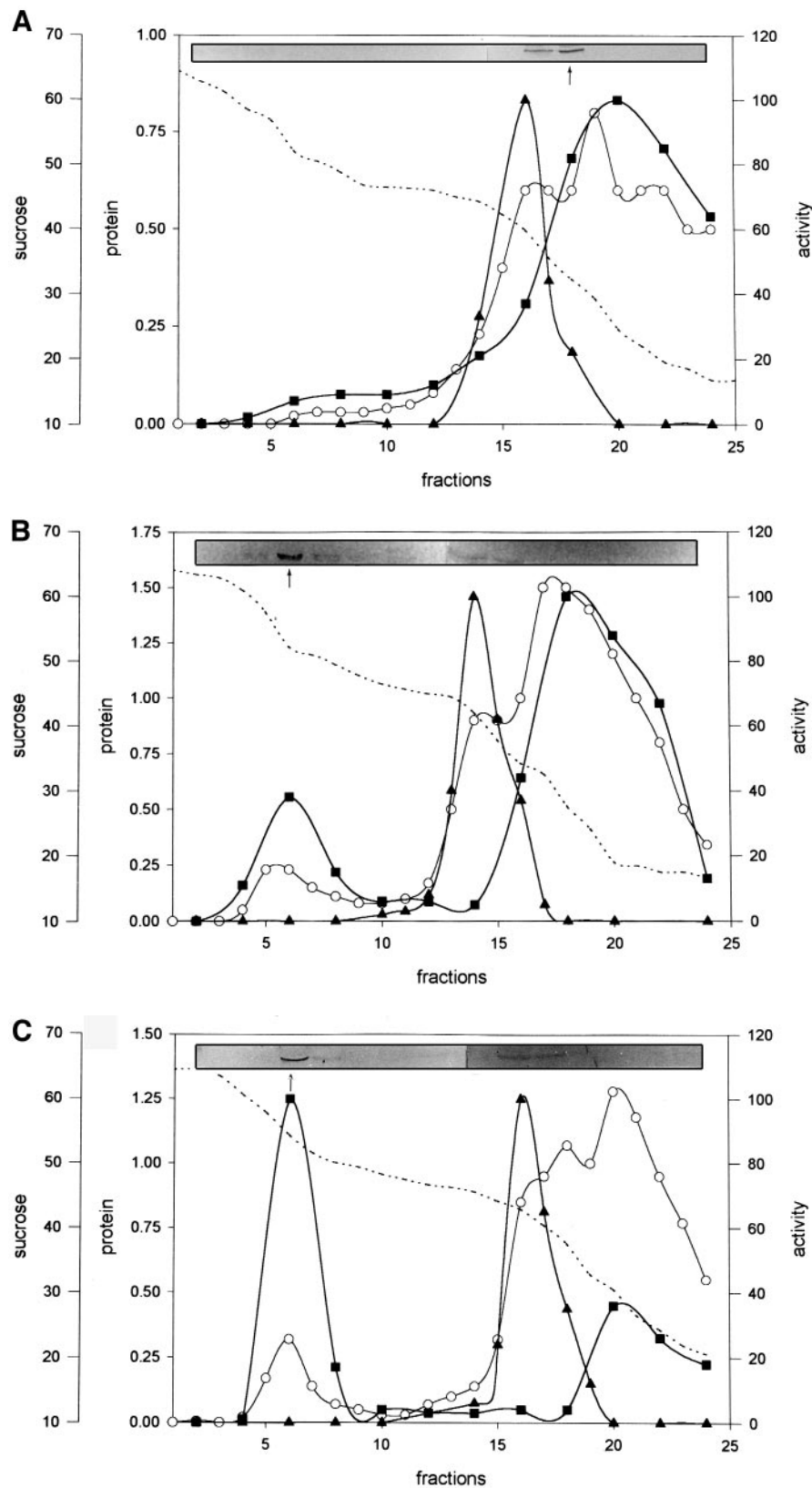
**HpPex4p is not essential for the selective degradation of whole peroxisomes in *H. polymorpha***

We investigated whether HpPex4p was essential for selective degradation of peroxisomes in methanol-induced *H. polymorpha* cells. To this end, cells of  $\Delta pex4::P_{AOX}PEX5^{4\times}$  were induced for 18 h on methanol and subsequently exposed to glucose excess conditions using identically grown wild-type cells as control. Biochemical analyses revealed that the patterns of the decrease in AO protein and the peroxisomal membrane protein Pex14p were similar in  $\Delta pex4::P_{AOX}PEX5^{4\times}$  and wild-type control cells (data not shown). These results indicated that HpPex4p is not essential for the selective degradation of whole peroxisomes in *H. polymorpha*.

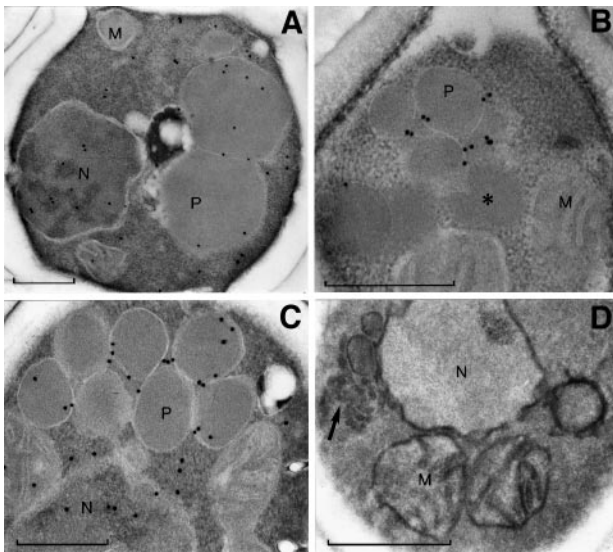
## Discussion

Here we report the isolation and characterization of the *H. polymorpha* PEX4 gene. Its protein product, HpPex4p, belongs to the UBC enzyme family which also includes the *P. pastoris* and *S. cerevisiae* Pex4p orthologues. We showed that deletion of *H. polymorpha* PEX4 led to a specific import defect for matrix proteins which require the PTS1 receptor HpPex5p for import. The PTS2 protein AMO is, however, properly imported in the absence of HpPex4p. Also, peroxisomal membrane proteins are inserted normally into the organellar membrane in  $\Delta pex4$  cells. These data suggest that the PTS1 protein import machinery of *H. polymorpha* involves ubiquitin conjugation to an as yet unknown protein catalysed by HpPex4p. Whether PpPex4p and ScPex4p are also involved predominantly in PTS1 protein import is not fully clear from the literature data. Gould *et al.* (1992) reported a major block in catalase import in the original *pex4* mutant of *P. pastoris* (*pas4-1*), but the subcellular location of a PTS2 protein was not studied in this mutant (Gould *et al.*, 1992). Differential centrifugation studies on an *S. cerevisiae* PEX4 null mutant suggested that these cells are impaired both in CAT (PTS1) and thiolase (PTS2) import, but the defect in CAT, compared with thiolase, appeared to be much more severe (Wiebel and Kunau, 1992). However, since AMO is the sole PTS2 protein identified so far in *H. polymorpha* and present at relatively low amounts, it cannot be excluded that HpPex4p may also be required for import of other PTS2 proteins as it is for import of baker's yeast thiolase.

In wild-type *H. polymorpha*, the amount of HpPex4p



**Fig. 9.** Cell fractionation of glycerol/methanol/ammonium sulfate-grown  $\Delta pex4$  (A),  $\Delta pex4::P_{AOX}PEX5^{4\times}$  (B) and wild-type cells (C). Sucrose density gradients were prepared from post-nuclear supernatants. The dotted lines show the sucrose concentration pattern, and the protein concentration pattern ( $\circ$ ), and the distributions of the activities of the peroxisomal marker AO ( $\blacksquare$ ) and mitochondrial cytochrome *c* oxidase ( $\blacktriangle$ ) are also shown. The cytosolic portion of AO in the gradient of wild-type *H. polymorpha* (C) most likely represents AO protein leaked from damaged organelles. A similar phenomenon may explain the relatively high portion of soluble AO protein in (B). The Western blots show the distribution of Pex3p in the even fractions of the gradient. Equal portions of the fractions were used. The Pex3p peak fractions are indicated by an arrow. Sucrose is expressed as percentage w/w, protein as mg/ml. The enzyme activities are expressed as percentages of the peak activities, which were arbitrarily set at 100.

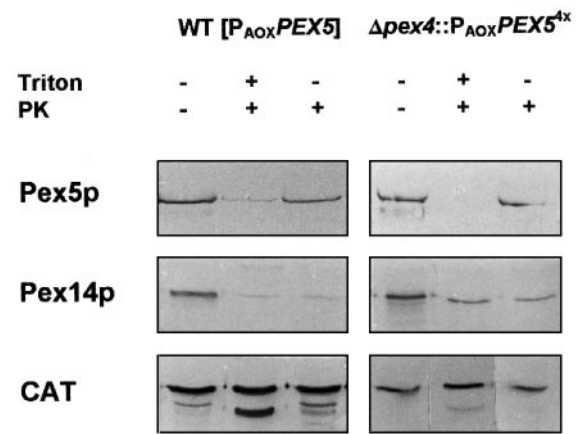


**Fig. 10.** Localization of Pex5p in wild-type and Pex5p-overproducing strains. (A) Methanol-grown wild-type cells; (B) methanol-induced  $\Delta pex4::P_{AOX}PEX5^{2\times}$  cells; (C) methanol-induced  $\Delta pex4::P_{AOX}PEX5^{4\times}$  cells; (D)  $\Delta pex4$  cells, overproducing Pex10p, showing that the enhanced amounts of Pex10p do not influence the peroxisomal defect in  $\Delta pex4$  cells (arrow = peroxisomal membrane remnants). The electron micrographs in (A–C) were of cells fixed in glutaraldehyde ( $\alpha$ -Pex5p/GAR-gold, uranylacetate), the one in (D) was of a  $KMnO_4$ -fixed cell. M, mitochondrion; N, nucleus; P, peroxisome. Bar = 0.5  $\mu m$ .

was invariably low. Expression studies indicated that these low Pex4p levels are due to the very weak *PEX4* promoter. These studies also revealed that *PEX4* is not induced during growth of cells on methanol. The low levels of Pex4p in *H. polymorpha* hampered the subcellular localization of this protein by immuno(cyto)chemical methods. Previous research on *S. cerevisiae* and *P. pastoris* localized Pex4p to peroxisomes in these species (Wiebel and Kunau, 1992; Crane *et al.*, 1994). Upon *PEX4* overexpression, HpPex4p was predominantly cytosolic, while a portion may be present in large protein complexes or bound to membrane structures. These structures are most likely not peroxisomes because in sucrose gradients HpPex4p was not detectable in peroxisomal peak fractions. However, since HpPex4p overproduction may alter its location, an unequivocal conclusion could not be drawn.

The molecular mechanisms of the function of HpPex4p in peroxisomal protein import are not yet fully clear. Pex4p belongs to the UBC family of enzymes, which are known to catalyse the second step in protein ubiquitination. In this process, ubiquitin first binds to an activator enzyme which transfers it to the conserved cysteine in the active site of the UBC enzyme. A ligase subsequently is required to bind ubiquitin to its target protein. In all three yeast Pex4ps, the UBC active site cysteine is conserved. For PpPex4p and ScPex4p, it has been shown that this cysteine residue is indeed essential for the function of the protein in peroxisome biogenesis (Wiebel and Kunau, 1992; Crane *et al.*, 1994). Moreover, conjugation of ubiquitin to *P. pastoris* Pex4p has also been demonstrated (Crane *et al.*, 1994). Therefore, it is likely that the yeast Pex4 proteins function as UBC enzymes.

Ubiquitin conjugation has been implicated in various cellular processes (reviewed by Hochstrasser, 1996). Of



**Fig. 11.** Protease protection assay using an organellar pellet prepared from methanol-induced cells of wild-type [*P<sub>AOX</sub>PEX5*] (A) and  $\Delta pex4::P_{AOX}PEX5^{4\times}$  (B). Equal amounts of protein were incubated with proteinase K (PK; 0.1 mg/ml) in the absence (–) or presence (+) of 0.1% Triton X-100 for 30 min at 0°C. Western blots were decorated using  $\alpha$ -Pex5p,  $\alpha$ -Pex14p or  $\alpha$ -CAT antibodies. The controls show that the matrix protein CAT is only partially cleaved in the presence of PK and Triton X-100. Pex14p degradation is dependent on PK, but independent of the presence of Triton X-100.

these, the ubiquitin–proteasome pathway is responsible for the specific and rapid degradation of a wide variety of cytosolic proteins by the proteasome. Ubiquitination also plays a role in the delivery of specific plasma membrane proteins to the vacuole, where these proteins subsequently are degraded. Ubiquitin conjugation is, however, not associated solely with proteolytic degradation, as was demonstrated by Chen *et al.* (1996) who presented evidence for the activation of a protein kinase by ubiquitination.

We demonstrated that HpPex4p does not play a role in the glucose-induced selective degradation of peroxisomes. However, HpPex4p may be involved in the turnover of specific peroxisomal proteins, including peroxins. In this scenario, HpPex4p may ubiquitinate components of non-functional protein complexes which are essential for peroxisomal protein import (like degradation of non-functional SecY complexes by FtsH in *Escherichia coli*; Akiyama *et al.*, 1996). We recently provided evidence that the protein components of these putative complexes must be present in relatively fixed stoichiometric amounts (Baerends *et al.*, 1997a,b). It can easily be envisaged that, with different half-lives of the constituent proteins, different regimes for removing these components may exist. Hence, the absence of Pex4p may lead to the accumulation of non-functional protein import complexes. However, this possibility is not very likely since it does not explain how the overproduction of solely PTS1 receptor molecules could enhance the import when the translocation machinery is hampered due to malfunction of individual components. The possibility that the PTS1 receptor is unstable in the absence of HpPex4p, a phenomenon which was observed for human Pex5p in several peroxisome-deficient cell lines (Dodt and Gould, 1996), is also not likely because Pex5p is normally present in methanol-induced *H. polymorpha*  $\Delta pex4$  cells, even at enhanced levels compared with wild-type cells. For this reason, we interpreted the increased levels of other peroxins implicated in protein import (Pex3p, Pex14p) in  $\Delta pex4$  cells as

a response of the organism to compensate for the import defect, caused by the defect in HpPex4p, rather than reflecting a decrease in their turnover rates. A comparable response has been observed in *H. polymorpha rif1* cells in which peroxisomal protein import was hampered due to limitation of the AO protein co-factor FAD (Evers *et al.*, 1994).

The finding that the same subset of proteins, namely PTS1 proteins and MS, are mislocated in  $\Delta pex4$  and  $\Delta pex5$  cells indicates that the function of HpPex4p may be related to that of HpPex5p. This view is strengthened by the observation that overproduction of Pex5p suppresses the protein import defect in  $\Delta pex4$  cells. An attractive explanation for the latter finding is that HpPex4p may be involved in recycling of Pex5p molecules. Dodt and Gould (1996) elegantly demonstrated that Pex5p cycles between peroxisomes and the cytosol in human skin fibroblasts. Based on the presence of Pex5p in both the cytosol and the peroxisomal matrix in wild-type *H. polymorpha* (van der Klei *et al.*, 1995), we speculated that cycling includes passage of Pex5p through the peroxisomal matrix. In the latter model, cytosolically formed Pex5p–PTS1 protein complexes enter the peroxisomal lumen, followed by dissociation and recycling of Pex5p (van der Klei and Veenhuis, 1996). Interestingly, in cells of complementation group 7 of human peroxisome biogenesis disorders, human Pex5p accumulates inside peroxisomes (Dodt and Gould, 1996). According to the Pex5p recycling model proposed for *H. polymorpha* Pex5p (van der Klei and Veenhuis, 1996), this phenotype could be caused by a block in Pex5p export. Also, the finding that Pex5p accumulates at the inner surface of the peroxisomal membrane in *H. polymorpha pex4* is consistent with the view that in this mutant recycling of Pex5p from the peroxisome to the cytosol is affected. However, the import–export model still lacks definite experimental proof, and at present we cannot rule out the possibility that Pex5p found in the peroxisomal matrix of wild-type *H. polymorpha* represents dead-end molecules and consequently that recycling of Pex5p takes place from the organellar surface. However, in both scenarios, the low rate of Pex5p-dependent import in  $\Delta pex4$  cells can then be explained readily as being due to the relatively low abundance of the cytosolic Pex5p relative to the high amount of newly synthesized PTS1 matrix proteins. Upon overproduction of Pex5p, the limiting amount of cytosolic Pex5p is then replenished, resulting in an increase of PTS1 protein import in  $\Delta pex4$  cells.

The concept of Pex5p as a cycling receptor implies its continuous binding to and dissociation from other proteins (e.g. cargo proteins, the putative docking proteins Pex13p and Pex14p). Pex4p-mediated ubiquitin conjugation to one of these proteins could be necessary to dissociate the Pex5p–protein complex in order to allow recycling back to the cytosol. In order to test this hypothesis, it is of major importance to identify the substrate(s) of Pex4p. This topic currently is being addressed in our laboratory.

## Materials and methods

### Strains, media and growth conditions

The *H. polymorpha* strains used in these studies are *per2-1* [*leu1.1 per2-1*] (Titorenko *et al.*, 1993), NCYC 495 [*leu1.1 ura3*] and  $\Delta pex4::URA3$  [*leu1.1*  $\Delta pex4$ ]. The strains were grown at 37°C on YPD medium

containing 1% yeast extract, 2% peptone and 2% glucose, on minimal media containing 0.67% yeast nitrogen base (Difco) or on mineral medium (van Dijken *et al.*, 1976). Glucose (0.5%), methanol (0.5%) or a glycerol/methanol (0.1%/0.5%) mixture were added as carbon source, with ammonium sulfate (0.2%) or methylamine (0.2%) as nitrogen source. When required, amino acids and uracil were added to a final concentration of 30 µg/ml. For cloning purposes, *Escherichia coli* DH5 $\alpha$  was used and grown as described (Sambrook *et al.*, 1989).

### Isolation and characterization of the PEX4 gene

Standard recombinant DNA techniques (Sambrook *et al.*, 1989) and genetic manipulations of *H. polymorpha* (Faber *et al.*, 1992, 1994) were performed as detailed before. Mutant *per2-1* was used to clone the corresponding gene by functional complementation essentially as described by Baerends *et al.* (1996). The complementing genomic DNA fragment was cloned as a 2.0 kb Klenow-treated *EcoRI*–*NheI* fragment into the *SmaI* site of pBluescriptII SK<sup>+</sup> (Stratagene Inc., San Diego, CA) in two orientations. Sequencing was carried out using an automatic sequencer and the *Taq* dye deoxy terminator Cycle Sequencing Kit (Applied Biosystems 373A). DNA and amino acid sequence analyses were done using the PC-GENE™ program release 6.70 (Intelligenetics, Mountain View, CA). The TBLASTN algorithm (Altschul *et al.*, 1990) was used to search the DDBJ/EMBL/GenBank database (release of July 9, 1997) for DNA and protein sequences showing similarity to *PEX4* and its protein product.

### Disruption of the PEX4 gene

A disruption construct was made by cloning a 2.0 kb Asp718 (Klenow-treated) *BamHI* *URA3* fragment of *H. polymorpha* (Merkelbach *et al.*, 1993) between the T4 polymerase-treated *PvuI* site and the *BglII* site (located in the *PEX4* ORF) of the 2.0 kb complementing fragment in pBluescriptII SK<sup>+</sup>. The disruption cassette was isolated by digestion with *NruI* and *HpaI* and transformed to *H. polymorpha* NCYC495 *leu1.1 ura3*. Methanol utilization-deficient colonies (Mut<sup>−</sup>) were selected, and proper integration of *URA3* was determined by Southern blot analysis using the ECL direct nucleic acid labelling and detection system (Amersham Corp., Arlington Heights, IL; data not shown). Selected  $\Delta pex4::URA3$  strains were tested for complementation with the original complementing fragment. Segregation and complementation analyses of the  $\Delta pex4$  mutant were performed as described previously (Titorenko *et al.*, 1993).

### Construction of Pex4p- and Pex5p-overproducing strains

A *HindIII* site was introduced in front of the *PEX4* start codon by PCR using primer 5′-TTTGCAAGCTTCATGTCTTCTACAGAAAAGC-3′. Subsequently, the *PEX4* ORF was cloned as a *BamHI* (Klenow-treated)–*HindIII* fragment in vector pHIPX4 (Gietl *et al.*, 1994) digested with *SmaI* and *HindIII*. The resulting plasmid, carrying the *PEX4* gene under control of the AO promoter (*P<sub>AOX</sub>*), was linearized with *StuI* and transformed into  $\Delta pex4::URA3$ . Proper integration of the plasmid at the *P<sub>AOX</sub>* locus was determined by Southern blot analysis (data not shown). Two strains were selected carrying either two or multiple (most probably five) copies of the *P<sub>AOX</sub>PEX4* cassette.

To obtain overexpression of *PEX5* in  $\Delta pex4$  cells, *StuI*-linearized plasmid pHIPX4-*PEX5* (van der Klei *et al.*, 1995), carrying the *PEX5* gene under control of the *P<sub>AOX</sub>*, was transformed to  $\Delta pex4::URA3$ . Proper integration of the plasmid at the *P<sub>AOX</sub>* locus was analysed by Southern blotting (data not shown). Three strains were selected carrying either one, two or multiple (most probably four) copies of the *P<sub>AOX</sub>PEX5* cassette. As a control, wild-type *H. polymorpha* containing *P<sub>AOX</sub>PEX5* was used (van der Klei *et al.*, 1995).

### Expression of the PEX4 gene

The activity of the *PEX4* promoter (*P<sub>PEX4</sub>*) was studied using  $\beta$ -lactamase as reporter enzyme.  $\beta$ -Lactamase activity was determined in an *H. polymorpha* strain carrying a single copy of an in-frame fusion of the  $\beta$ -lactamase gene and *P<sub>PEX4</sub>*. As controls, similar constructs were made for *P<sub>AOX</sub>* and the promoter of *PEX8* (*P<sub>PEX8</sub>*). To enable in-frame cloning of the  $\beta$ -lactamase gene behind *P<sub>PEX4</sub>*, an *EcoRI* site was introduced after the ATG start codon of *PEX4* with PCR using primer 5′-CCTTCAGAAATTCGCTTTTCTGTAGAA-GAC-3′. Subsequently, *P<sub>PEX4</sub>* was ligated as a *NotI*–*EcoRI* fragment, together with the  $\beta$ -lactamase gene as an *EcoRI*–*HindIII* fragment, into pBluescriptII SK<sup>+</sup>, treated with *NotI* and *HindIII*. From the resulting plasmid, the expression cassette was isolated as a *BamHI*–*HindIII* fragment, treated with Klenow enzyme and ligated into the unique *SmaI* site of pHII (pHII was constructed by ligating the

*URA3* gene of *H. polymorpha* as a blunt-ended 2.3 kb *Bam*HI fragment into the unique *Nde*I site of pUC19). The resulting plasmid, pX-PEX4, carried the  $\beta$ -lactamase gene under control of the  $P_{PEX4}$ . The expression cassette containing the gene encoding  $\beta$ -lactamase control of  $P_{AOX}$  was isolated from pHIPX3- $\beta$ lac (Waterham *et al.*, 1994) as a *Bam*HI-*Bgl*II fragment and cloned into the *Bam*HI site of pH11, resulting in plasmid pX-AOX. The expression cassette with the  $\beta$ -lactamase gene under control of  $P_{PEX8}$  was isolated from plasmid PHIPX3- $P_{PEX8}$ - $\beta$ lac (Waterham *et al.*, 1994) as a *Bam*HI-*Asp*718 fragment, ligated into pH11 and treated with *Bam*HI and *Asp*718, resulting in plasmid pX-PEX8. The  $\beta$ -lactamase expression plasmids were linearized in the *URA3* gene using the unique *Apa*I site and transformed to *H. polymorpha* NCYC 495 *leu1.1 ura3*. Correct, single copy integration in the *URA3* locus was checked by Southern blotting (data not shown).

### Biochemical methods

Crude extracts were prepared as described by Waterham *et al.* (1994). AO and AMO were assayed as described by Verduyn *et al.* (1984), CAT (Lück, 1963), cytochrome *c* oxidase (Douma *et al.*, 1985) and  $\beta$ -lactamase activities (Waterham *et al.*, 1994) were determined as described previously. Cell fractionation was performed as detailed by van der Klei *et al.* (1998). Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as a standard. SDS-PAGE was carried out as described (Laemmli, 1970). Western blotting was performed according to Kyhse-Andersen (1984) and blots were decorated using specific polyclonal antibodies against various *H. polymorpha* peroxisomal proteins. The antibodies against *S. cerevisiae* Sec63, which cross-reacted with the *H. polymorpha* Sec63 orthologue, were a gift from Dr R. Schekman, Berkeley, CA. For generation of antisera recognizing HpPex4p, a synthetic peptide, consisting of amino acids 85–90 and 13–23 of HpPex4p, was coupled to keyhole limpet haemocyanin and used to immunize rabbits. Organellar pellets were subjected to PK treatment as described by Komori *et al.* (1997).

### Electron microscopy

Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously (Waterham *et al.*, 1994). Morphometric analysis on  $KMnO_4$ -fixed cells was performed as described by Veenhuis *et al.* (1978). Immunolabelling was performed on ultrathin sections of uncryl-embedded cells, using specific antibodies against various *H. polymorpha* proteins and gold-conjugated goat anti-rabbit (GAR-gold) antibodies according to the instructions of the manufacturer (Amersham Corp., Arlington Heights, IL).

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