Overproduction of Pex5p Stimulates Import of Alcohol Oxidase and Dihydroxyacetone Synthase in a Hansenula polymorpha pex14 Null Mutant*

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Hansenula polymorpha Δpex14 cells are affected in peroxisomal matrix protein import and lack normal peroxisomes. Instead, they contain peroxisomal membrane remnants, which harbor a very small amount of the major peroxisomal matrix enzyme alcohol oxidase (AO) and dihydroxyacetone synthase (DHAS). The bulk of these proteins is, however, mislocated in the cytosol. Here, we show that in Δpex14 cells overproduction of the PTS1 receptor, Pex5p, leads to enhanced import of the PTS1 proteins AO and DHAS but not of the PTS2 protein amine oxidase. The import of the PTS1 protein catalase (CAT) was not stimulated by Pex5p overproduction. The difference in import behavior of AO and CAT was not related to their PTS1, since green fluorescent protein fused to the PTS1 of either AO or CAT were both not imported in Δpex14 cells overproducing Pex5p. When produced in a wild type control strain, both proteins were normally imported into peroxisomes. In Δpex14 cells overproducing Pex5p, Pex5p had a dual location and was localized in the cytosol and bound to the outer surface of the peroxisomal membrane. Our results indicate that binding of Pex5p to the peroxisomal membrane and import of certain PTS1 proteins can proceed in the absence of Pex14p.

Most organellar proteins in eukaryotic cells are synthesized on free or membrane-bound cytosolic ribosomes and sorted to their final destination by unique targeting signals. These signals are recognized by specific receptor proteins, which are either components of the cytosol (e.g. signal recognition particle for the endoplasmic reticulum (1) or importin α for nuclear proteins (2)) or are bound to the membrane of the target organelle (e.g. Tom proteins at the mitochondrial outer membrane (3)). Following recognition of the targeting signal and subsequent routing of the receptor-cargo complex to the actual import site, the protein is transported into the organelle. Generally, proteins are released from their receptors prior to import. However, in the case of nuclear protein import the receptor dissociates after transport through the nuclear pore complex, followed by export of the receptor back to the cytosol (3).

For peroxisomal matrix proteins comparable mechanisms may exist (4). Sorting of these proteins is mediated by specific targeting signals, termed PTS1 and PTS2. The PTS1 is located at the extreme carboxyl terminus of most peroxisomal matrix proteins and consists of the tripeptide SKL-COOH or a conserved variant thereof (5–7). In the methylo trophic yeast Hansenula polymorpha, the key enzymes of methanol metabolism, alcohol oxidase (AO), dihydroxyacetone synthase (DHAS), and catalase (CAT), all contain a PTS1 (8, 9). The PTS2, a nonapeptide with the consensus RLX₅H/Q₉L, is located at the amino terminus and observed for a limited number of matrix proteins (7, 10, 11). Typical examples of these are peroxisomal thiolases (12, 13) and H. polymorpha amine oxidase (14).

The PEX5 gene encodes the receptor for PTS1 proteins, Pex5p (15–17). Yeast PEX5 null mutants (Δpex5) are characterized by a complete block of PTS1 protein import. In such mutants the PTS2 protein import machinery is normally functioning, which indicates that both pathways can act independently in yeast. This was confirmed by the finding that in PEX7 null mutants, which lack the PTS2 receptor Pex7p, PTS2 proteins are mislocated in the cytosol, whereas PTS1 proteins are still properly imported (18, 19).

Pex5p binds the PTS1 of newly synthesized peroxisomal matrix proteins upon their synthesis in the cytosol. Subsequently, this receptor-cargo complex is thought to interact with proteins at a putative Pex5p docking site at the peroxisomal membrane (11, 20). Peroxins proposed to be involved in the docking and subsequent translocation process include Pex13p (21–23), Pex14p (24–26), and Pex17p (27). The absence of either one of these proteins results in defects in both PTS1 and PTS2 protein import. In various genetic and biochemical studies a direct interaction of Pex5p with Pex13p and Pex14p has been demonstrated (21, 22, 25, 26, 28). Moreover, interactions between Pex13p and Pex14p and between Pex14p and Pex17p have been reported (27).

Our current working model of PTS1 protein import in H. polymorpha includes that in the Pex5p-cargo complex is translocated across the peroxisomal membrane into the organelar matrix, followed by dissociation and subsequent export of Pex5p from the matrix back to the cytosol (4). This model is based on the finding that in this organism Pex5p is present both inside peroxisomes and the cytosol (17). Also, evidence has been obtained that Pex4p, a ubiquitin-conjugating enzyme, plays a crucial role in recycling of Pex5p back to the cytosol (29). This was evident from the finding that overexpression of PEX5 suppresses the PTS1 protein import defect in H. poly-

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† The abbreviations used are: AO, alcohol oxidase; DHAS, dihydroxyacetone synthase; CAT, catalase; GFP, green fluorescent protein; WT, wild type; kb, kilobase pair.
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### TABLE I

| H. polymorpha strains | Relevant properties | Source or Ref. |
|-----------------------|---------------------|---------------|
| CBS 4732              | Wild type (WT)      | This study    |
| WT·: P::AOXGFP.SKI    | CBS 4732 with integration of plasmid pHIPZ4-GFP.SKI at the P::AOX locus | This study |
| Δpex1 ·: P::AOXPEX5"   | PEX1 deletion strain, leu1.1 | 40 |
| Δpex3 ·: P::AOXPEX5"   | PEX3 deletion strain, leu1.1 | 41 |
| Δpex6 ·: P::AOXPEX5"   | PEX6 deletion strain, leu1.1 | 40 |

| Strains/plasmids       | Relevant properties | Source or Ref. |
|------------------------|---------------------|---------------|
| pBluescript II KS +    | pBluescript II KS + containing the zeocine selection cassette from pPICza | This study |
| pFEM39                 | pBluescript II KS + containing the zeocine selection cassette from pPICza | Stragagene, La Jolla, CA |
| pEGFP-C1               | This study          | Invitrogen, Groningen, the Netherlands |
| pHIPX4                 | This study          | This study |
| pHIPX4-PEX5            | pHIPX4 containing the PEX5 gene under control of the alcohol oxidase promoter (P::AOX) | 17 |
| pHIPX4-URA3-PEX5       | Plasmid pHIPX4-P::AOX containing the URA3 gene | This study |
| pHIPZ4                 | pBluescript II KS + -derived vector containing the alcohol oxidase promoter region (P::AOX), the amino oxidase terminator region (T::AMO), and the Zeocine resistance (Zeo r) marker | This study |
| pHIPZ4-GFP.SKI         | pHIPZ4 containing the GFP gene fused to the tetrapeptide SKI under control of the alcohol oxidase promoter (P::AOX) | This study |
| pHIPZ4-GFP.LARF        | pHIPZ4 containing the GFP gene fused to the tetrapiptide LARF under control of the alcohol oxidase promoter (P::AOX) | This study |

**Overexpression of PEX5 in H. polymorpha Δpex1 cells.** Remarkably, in these cells a significant portion of Pex5p accumulated at the inner surface of the peroxisomal membrane (29). A likely explanation for these observations is that the recycling of Pex5p is hampered in the absence of Pex4p thus releasing the import of newly synthesized matrix proteins dependent on newly formed Pex5p.

In this paper we show that, like in Δpex4 cells, overexpression of PEX5 also significantly stimulates PTS1-protein import in Δpex14 cells. In Δpex14 cells overexpressing PEX5 significant amounts of Pex5p accumulate at the outer surface of the peroxisomal membrane, which suggested that (under these conditions) Pex14p is not necessary for the association of Pex5p with the peroxisomal membrane.

**MATERIALS AND METHODS**

**Organisms and Growth Conditions.**—The H. polymorpha strains used in this study are listed in Table I. H. polymorpha was grown at 37 °C in YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (w/v) glucose or in mineral medium (30) supplemented with 0.5% (w/v) glucose or 0.5% (v/v) methanol as carbon source and 0.25% (w/v) ammonium sulfate or methylamine as nitrogen source.

**Construction of Pex Mutants Overexpressing PEX5—** H. polymorpha Δpex1, Δpex3, Δpex6, pex10.1, and Δpex14 mutants were transformed with StuI-linearized plasmid pHIPX4-PEX5 (Table I) using electroporation (31). Site-directed integration of the plasmids at the P::AOX locus was confirmed by Southern blot analysis. Strains containing multiple copies of the pHIPX4 derivatives were selected as reported earlier (29).

**Construction of pHIPZ4—** First the zeocine selection cassette was taken from pPICza (Table I) as a 1.1-kb MluI fragment and inserted into Asp718 (Klenow fill-in of sticky ends)-BamHI-digested pBluescript II KS + (Table I), resulting in pFEM39. Subsequently, a 2.0-kb NotI-BglII fragment, containing the amino oxidase promoter region (P::AOX) and amine oxidase terminator region (T::AMO), was taken from pHIPX4 (Table I) and inserted into NotI-BamHI-digested pFEM39, resulting in pHIPZ4.

**Construction of Strains Producing PTS1-containing GFP Proteins—** The chimeric gene encoding GFP fused to the targeting signal of AO (LARF-COOH) was constructed by polymerase chain reaction-mediated amplification of the GFP gene of pEGFP-C1 (Table I) using the following oligonucleotides (oligonucleotides were obtained from Life Technologies, Inc.): upstream 5'-AGA AAG CTT ATG GTG AGC AAG GGC-3' (underlined site underlined and downstream 5'-CCC GTC GAC TTA GAA TCT GGC CAG CTT GTA GAG CTC GTC-3' (SalI site underlined and the nucleotides that code for the tetrapeptide LARF are depicted in italics). For the construction of the gene encoding GFP fused to the targeting signal of CAT (SKI-COOH) the following primers were used: upstream 5'-CCG GAA TCC ATG AGC AGC GAG GCC-3' (HindIII site underlined) and downstream 5'-CCG TCT CAG TTA OAT CTT CGA CTA GAG CTC GTC-3' (SalI site underlined and the nucleotides that code for the tetrapeptide SKI are depicted in italics).
FIG. 1. Overall morphology of methanol-induced H. polymorpha cells to show the re-introduction of peroxisomes in Δpex14 cells, which overproduce Pex5p. In methanol-grown WT control cells normal peroxisomes are present (A). Peroxisome development is not restored in pex10−1::pAOXPEX5 cells incubated for 24 h in methanol containing media (B) (arrow, peroxisomal remnants; arrow, AO aggregate). Methanol-induced Δpex14 cells contain peroxisomal remnants (C) (arrow, peroxisomal remnants). However, in identically grown Δpex14::pAOXPEX5 cells several peroxisomes are present (D). Also, when lower Pex5p levels are obtained in Δpex14, i.e. in Δpex14::PpAOXPEX5 grown for 16 h on glycerol, several small peroxisomes (arrow) are observed in the cells (E). Electron micrographs are taken of KMnO4-fixed cells, poststained with uranyl acetate. The abbreviations used are as follows: M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The marker represents 0.5 μm.

FIG. 2. Western blot showing the level of Pex5p in crude extracts of methanol-grown WT (lane 1), methanol-induced Δpex14 (lane 2), Δpex14::pAOXPEX5 (lane 3), and Δpex14::PpAOXPEX5 (lane 4) using a-Pex5p antibodies. Compared with WT and Δpex14 extracts, Δpex14::PpAOXPEX5 and Δpex14::PpAOXPEX5 extracts contained enhanced amounts of Pex5p.

(Invitrogen, Groningen, The Netherlands). Site-directed integration of the plasmid at the PpAOX locus was confirmed by Southern blot analysis.

Biochemical Methods—Alcohol oxidase (32), catalase (33), and cytochrome c oxidase (34) were assayed as described. Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was carried out as described (35), and gels were subjected to Western blotting (36). Nitrocellulose blots were decorated using specific polyclonal antibodies against various H. polymorpha proteins. Cell fractionation (37), flotation centrifugation (38), and proteinase K protection assays (29) were performed as described before.

Electron Microscopy—Cells were fixed and prepared for electron microscopy as described previously (39). Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific antibodies against various proteins and gold-conjugated goat anti-rabbit antibodies (39).

RESULTS

Peroxisome Formation Is Restored in Δpex14 Cells Overproducing Pex5p—Previously, we observed that overexpression of PEX5 restored the formation of peroxisomes in H. polymorpha Δpex4 cells (29). To determine whether this phenomenon also occurs in other H. polymorpha pex mutants, a PEX5 overexpression cassette (PpAOXPEX5) was integrated in two or multiple (3–5) copies into the genomes of Δpex1, Δpex3, Δpex6, pex10−1, and Δpex14 (Table I). Cells of these strains were incubated for 24 h in methanol-containing media to induce the PpAOX and the major peroxisomal PTS1 proteins AO and DHAS. None of the strains showed significant growth on methanol indicating that full restoration of peroxisome formation did not occur in any of the strains. We subsequently examined thin sections of KMnO4-fixed cells from these cultures for the presence of peroxisomal structures. Methanol-grown WT cells, used as a control, characteristically contain several large peroxisomes (Fig. 1A). In Δpex1, Δpex3, Δpex6, and pex10−1 cells overexpressing PEX5, such organelles were invariably absent (Fig. 1B, pex10−1; others not shown). However, in Δpex14::PpAOXPEX5 cells the presence of several peroxisomes was evident. The size of these organelles was slightly reduced compared with those present in WT control cells (Fig. 1D).

In order to compare the Pex5p levels in WT, Δpex14 and Δpex14 overproducing Pex5p Western blotting experiments were performed. These experiments revealed that methanol-induced WT and Δpex14 cells contain similar levels of Pex5p. The Pex5p level was, however, significantly increased in strains containing two or multiple copies of the PpAOXPEX5 overexpression cassette (Fig. 2). Densitometric scanning of blots prepared from crude extracts of methanol-grown WT cells and Δpex14::PpAOXPEX5 cells induced for 24 h on methanol-containing media revealed that in Δpex14::PpAOXPEX5 the amount of Pex5p was approximately 100-fold higher than in WT control cells. Much lower overproduction of Pex5p (i.e. a 20-fold increase compared with WT cells) was obtained in a Δpex14 strain containing only two copies of the PpAOXPEX5 cassette (Δpex14::PpAOXPEX5) upon growth for 16 h on glycerol-containing medium.
pared from KMnO₄-fixed cells of this culture revealed that also a 20-fold increase in Pex5p levels resulted in the formation of small peroxisomes in H. polymorpha Δpex14 (Fig. 1E).

PTS1 Protein Import Is Stimulated by Pex5p Overproduction in Partially Restored Δpex14 Cells—In methanol-induced H. polymorpha Δpex14 cells normal peroxisomes are absent. Instead these cells contain peroxisomal membrane remnants that contain a very minor portion of AO and DHAS, whereas the bulk of these proteins is mislocated in the cytosol (24). Immunocytochemical analysis of glycerol-grown Δpex14:PAOXPEX5mc cells revealed that the small peroxisomes that are formed in these cells contain the PTS1 proteins AO (Fig. 3A) and DHAS (not shown). Similarly, the larger organelles present in methanol-induced Δpex14:PAOXPEX5mc cells are characterized by significant amounts of AO (not shown) and DHAS protein (Fig. 3B). Judged from the distribution of the labeling, a portion of these matrix proteins was still mislocated in the cytosol. By using antibodies against Pex3p, a peroxisomal membrane protein, labeling was obtained at the membrane of these organelles (Fig. 3C), indicating that they indeed represent peroxisomes. Remarkably, import of a third PTS1 protein, catalase (CAT), was not significantly stimulated by Pex5p overproduction (Fig. 3D). As expected, the location of the PTS2 protein amine oxidase remained cytosolic in Δpex14:PAOXPEX5mc cells (data not shown).

The immunocytochemical data were confirmed biochemically by cell fractionation experiments. To this end post-nuclear supernatants, prepared from methanol-grown WT cells or methanol-induced Δpex14 and Δpex14:PAOXPEX5mc cells, were subjected to sucrose density centrifugation (Fig. 4). In gradients prepared from WT cells, peroxisomes migrate to a high density corresponding to 54% sucrose (fraction 6, Fig. 4A), indicated by a distinct peak of AO and CAT activity. Also the peroxisomal membrane marker Pex3p is present in these fractions, as evident from Western blots prepared from the gradient fractions (inset in Fig. 4A). The AO and CAT activities detected in the top fractions of the gradient most likely are due to leakage of these proteins as a result of the fractionation procedure (37). In the gradient of Δpex14 cells (Fig. 4B) AO, CAT, and Pex3p are absent at high densities. Instead, AO and CAT activities are present at the top of the gradient, indicative for a cytosolic location. Pex3p migrated to a density corresponding to 37% sucrose (fraction 17, Western blot included in Fig. 4B). Previously, evidence was provided using immunocytochemistry that the peroxisomal membrane remnants, present in Δpex14 cells, contained Pex3p and very small amounts of AO and DHAS (24). Hence, these structures most likely migrated to a density corresponding to 37% sucrose (fraction 17) of the gradient prepared from Δpex14 cells. Gradients of methanol-induced Δpex14:PAOXPEX5mc cells again displayed a prominent peak of AO activity at 54% sucrose (fraction 7, Fig. 4C); also Pex3p was localized at this position (Western blot included in Fig. 4C). However, significant amounts of AO activity, exceeding those of WT controls, were also detected in the top fractions of this gradient (fraction 18–25) most likely representing the cytosolic portion of the AO protein demonstrated by immunocytochemistry (Fig. 3A). In contrast, CAT activity (Fig. 4C) and CAT protein, as determined by Western blotting (data not shown), was only detected in the top fractions of this gradient, thus confirming the immunocytochemical data that the import of CAT was not stimulated in Δpex14:PAOXPEX5mc cells.

To obtain additional evidence that the AO activity peak detected at 54% sucrose in the Δpex14:PAOXPEX5mc gradient indeed represented membrane-bound AO protein and was not due to the presence of dense AO protein aggregates and/or crystalloids, these fractions were subjected to floatation centrifugation. As shown in Fig. 5, both AO activity and the peroxisomal membrane protein Pex3p floated to lower densities and comigrated in a distinct protein peak at approximately 50% sucrose, indicating that AO is indeed membrane-bound.

Restoration of PTS1 Import Is Not Related to Variations in the PTS1—Overproduction of Pex5p in Δpex14 cells significantly stimulated import of the PTS1 proteins AO and DHAS.
but not of CAT. Possibly, this may be related to differences in affinities of *H. polymorpha* Pex5p for the PTS1 sequences of these proteins, i.e. LARF-COOH in case of AO, NKL-COOH in case of DHAS, and SKI-COOH in case of CAT (8, 9). To analyze this possibility, we constructed chimeric genes encoding green fluorescent protein (GFP) fused to the respective targeting signals of AO and CAT (GFP.LARF and GFP.SKI, respectively). These genes were introduced in Δpex14::PAOXPEX5mc and a WT control strain, and the location of their translation products was determined. In *H. polymorpha* WT cells the two GFP fusion proteins were efficiently targeted to the peroxisomes, as indicated by the punctate patterns observed by fluorescence microscopy (data not shown) and immunocytochemistry, using specific α-GFP antibodies (Fig. 6A, GFP.LARF and GFP.SKI, not shown). However, in Δpex14::PAOXPEX5mc cells, producing either GFP.LARF or GFP.SKI, a diffuse fluorescence pattern was observed, indicative for a cytosolic location (data not shown). To examine whether minor amounts of the fusion proteins may
be imported into peroxisomes, their location was also determined immunocytochemically. The labeling patterns, using α-GFP antibodies, revealed that both proteins were confined to the cytosol (Fig. 6, GFP.LARF and GFP.SKI, not shown).

Pex5p Accumulates in the Cytosol and at the Outer Surface of the Peroxisomal Membrane in Δpex14::PAOXPEX5mc Cells—In WT H. polymorpha Pex5p has a dual location and is present both in the cytosol and the peroxisomal matrix (Fig. 7); a similar location is found in WT cells overproducing Pex5p (not shown; see Ref. 17). In immunocytochemical experiments using ultrathin sections of methanol-induced Δpex14::PAOXPEX5mc cells, the bulk of Pex5p labeling was found at the cytosol. In addition, labeling was observed at the peroxisomal membrane but never on the organellar matrix (Fig. 7A). In sucrose gradients of a post-nuclear supernatant prepared from homogenized, methanol-induced Δpex14::PAOXPEX5mc cells, Pex5p sedimented at two specific locations. Pex5p cosedimented with peroxisomal marker proteins at 54% sucrose (fraction 7) and was also detected at top of the gradient where cytosolic proteins are located (fractions 18–23; Fig. 8A). Characteristically, the cytosolic Pex5p was predominantly detected at lower molecular weight due to proteolytic degradation. Taken together, these data indicate that
Protease protection analysis using purified peroxisomes from methanol-induced WT (A) and \textit{pex14} \textit{mc} cells (B).

Pex5p is present in both cytosolic and peroxisomal fractions in gradients prepared from \textit{pex14} \textit{mc} cells.

DISCUSSION

Pex14p is a peroxisomal membrane protein and is essential for peroxisome biogenesis (24–26, 44–46). The finding that Pex14p is able to bind Pex5p and Pex7p, the specific receptors for the PTS1 and PTS2, respectively (25), has led to the suggestion that Pex14p is a central component of the peroxisomal protein translocation machinery, most likely mediating the membrane docking event of these receptors. A similar function has been postulated for Pex13p; moreover, Pex13p and Pex14p have been shown to physically interact (21–23, 47). Pex13p and Pex14p are probably part of a protein complex, which is characterized by a rather fixed stoichiometry of its constituting components for normal functioning (24, 38). Recently, a potential third component of this complex, Pex17p, has been identified (27).
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Pex14p. The accompanying phenomenon, namely the accumulation of Pex5p at the peroxisomal membrane, could then be understood by assuming that Pex14p is in fact not the initial docking site of the Pex5p-cargo complex but functions at a later step of translocation (Fig. 10). It should be stressed that in WT H. polymorpha WT cells the presence of a PTS1 signal at the extreme carboxyl terminus is required and sufficient to direct a reporter protein to the peroxisomal matrix, a process that involves the function of the Pex5 receptor Pex5p (8, 9, 17). However, the differential import observed in Δpex14::P\textsubscript{AOX}P\textsubscript{EXS\textsubscript{5mc}} cells (e.g. AO compared with GFP.LARF), overproducing Pex5p, suggests that for normal protein location additional factors are also involved. These factors may be related to intrinsic properties of the corresponding proteins (e.g. in case of AO and DHAS) and/or may even include yet unknown “accessory” proteins (e.g. in case of CAT). The first option, mentioned above, is not only hypothetical since evidence has been obtained that specific peroxisomal matrix proteins contain such information. A relevant example of this is that the removal of the Pex5p from Pichia pastoris AO did only result in a partial block in import of the truncated protein (50). Another indication is the finding that in two-hybrid assays the Pex5p protein carnitine acetyltransferase from Saccharomyces cerevisiae interacts with Pex5p, also when the Pex5p is removed (51).

However, the finding that CAT is not imported in Δpex14::P\textsubscript{AOX}P\textsubscript{EXS\textsubscript{5mc}} cells lends support to the notion that the Pex5p import machinery is not uniform for all Pex5p proteins. A comparable phenomenon has been observed in case of Pex2 protein import in Yarrowia lipolytica; in this organism Pex2p is specifically involved in the import and oligomerization of the Pex2 protein thioloase (52). Other examples include Candida boidinii Pmp47 which is specifically required for import and assembly of DHAS (53) and FAD, which is essential for efficient import of AO in peroxisomes of H. polymorpha (54). Above this, variations may exist with respect to the specific requirements of import, e.g. related to the folding/oligomerization properties of imported proteins (import as monomer or oligomer), the energy requirements, or the kinetics of import. However, further experiments are required to elucidate the details of the Pex5p protein import machinery.

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