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Chapter 5

Re-assembly of peroxisomes in *Hansenula polymorpha* pex3 cells upon re-introduction of Pex3p involves the nuclear envelope

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Abstract

We analyzed the re-assembly of peroxisomes in *Hansenula polymorpha pex3* cells upon reintroduction of Pex3p. Within one hour after the onset of Pex3p production, a single organelle developed per cell, invariably in close proximity of the nuclear envelope. Subsequently, this organelle increased in size, migrated to a position in the vicinity of the cell wall, and multiplied by division. Fractionation experiments on homogenates of *pex3* cells, in which the endomembrane system was tagged with GFP, identified a small amount of GFP in peroxisomes present in the initial stage of peroxisome re-assembly. Taken together, our data suggest a distinct role for the endomembrane system in peroxisome re-assembly in complemented *pex3* cells.

Introduction

Peroxisomes are multi-functional organelles, which play an important role in cellular metabolism (for a review, see (De Duve, 1983; van der Klei and Veenhuis, 1997). Several peroxisome-associated disorders are identified in man, some of which are lethal (e.g. Zellweger syndrome; reviewed in (Wanders, 1999; Gould and Valle, 2000)). In yeasts, peroxisomes are generally involved in the primary metabolism of specific carbon and/or nitrogen sources used for growth (Veenhuis, 1992). Since peroxisomes lack DNA, all peroxisomal proteins, both soluble and membrane-bound, are post-translationally imported into the organelle (Fujiki et al., 1984).

We have isolated peroxisome-deficient (*pex*) mutants of the methylotrophic yeast *Hansenula polymorpha* and cloned seventeen of the corresponding genes. One of these, *PEX3*, encodes a 52 kDa protein essential for peroxisome biogenesis and maintenance (Baerends et al., 1996). The importance of Pex3p in peroxisome biogenesis is underlined by the absence of detectable peroxisomal membrane remnants (ghosts) in a *pex3* deletion strain, a characteristic that is shared only by the human *pex16* and *Saccharomyces cerevisiae pex19* mutant phenotypes (Honsho et al., 1998; Götte et al., 1998). All other *pex* mutants identified thus far contain ghosts. Although ghosts are missing in the *pex3* mutant, re-introduction and expression of the *PEX3* gene in such mutants resumes peroxisome biogenesis (Baerends et al., 1996; Ghaedi et al., 2000). The origin of the membrane of the newly formed organelles was still enigmatic. The classical model of peroxisome biogenesis involves growth and multiplication of existing organelles by fission, which implies that new peroxisomes develop from pre-existing ones (Lazarow and Fujiki, 1985). However, data that support the possibility of alternative pathways for peroxisome
biogenesis have accumulated recently (for a review, see Titorenko and Rachubinski, 2001). In this paper we describe that the re-introduction of normal peroxisomes in \textit{H. polymorpha} pex3 cells upon expression of the \textit{PEX3} gene proceeds rapidly and involves the nuclear envelope.

\section*{Materials and methods}

\subsection*{Microorganisms and growth conditions}
\textit{H. polymorpha} strains were grown in batch cultures at 37°C on mineral medium (van Dijken et al., 1976) supplemented with 0.5% carbon source (i.e. glucose or methanol) in the presence of 0.25% ammonium sulphate or ethylamine as nitrogen source. For growth on agar plates, all media were supplemented with 1.5% granulated agar. \textit{Escherichia coli} DH5α (\textit{supE}44 \textit{ΔlacU169} (\textit{φ}80\textit{lacZ}ΔM15) \textit{hsdR17 recA1 endA1 gyrA96 thi-1 relA1}) (Sambrook et al., 1989) was used for recombinant DNA procedures and was grown on LB-medium supplemented with the appropriate antibiotics.

\subsection*{DNA procedures}
\textit{H. polymorpha} was transformed by electroporation (Faber et al., 1994b). Recombinant DNA manipulations were performed essentially as described (Sambrook et al., 1989). Biochemicals were obtained from Roche (Almere, The Netherlands). Site-specific integration at the \textit{AMO}-locus was performed as outlined by Faber et al. (Faber et al., 1994a). Southern blotting was performed using the ECL direct nucleic acid labelling and detection system, as described by the manufacturer (Amersham Pharmacia Biotech, Little Chalfont, England). pHIPZ5-BiP\textsubscript{[1-30]}GFP was constructed as follows: The 1.8 kb \textit{SmaI}/\textit{SpeI} fragment of plasmid pFEM76 (Faber et al., 2002) was cloned into the 4.3 kb \textit{SalI}(Klenow-treated)/\textit{SpeI} fragment of pHIPZ6-Nia.

The \textit{H. polymorpha} pex3::\textit{pAOX}\textit{PEX3}::\textit{pAMO} BiP\textsubscript{[1-30]}GFP strain, which can be used to independently express the \textit{PEX3} and BiP\textsubscript{[1-30]}GFP open reading frames in a pex3 mutant background, was constructed as follows. NarI-linearized pZ5-BiP\textsubscript{[1-30]}GFP was used to transform \textit{H. polymorpha} pex3::\textit{pAOX}\textit{PEX3} (Baerends et al., 1997). Southern blot analysis of selected zeocin-resistant transformants was performed to verify proper site-specific integration of the expression cassette at the \textit{AMO}-locus in a single copy (data not shown).
Biochemical methods
Protoplasts were prepared and homogenized as described (van der Klei et al., 1998). Cell fractionation experiments were performed as outlined (van der Klei et al., 1998). Cytochrome c oxidase activities were determined as described (Douma et al., 1985). Protein concentrations were determined using the BioRad protein assay kit (BioRad GmbH, Munich, Germany), using bovine serum albumin as standard. SDS-PAGE and Western blotting were carried out as described (Laemmli, 1970; Kyhse-Andersen, 1984). Blots were decorated using the chromogenic (NBT-BCIP) or chemiluminiscent (POD) Western Blotting kit (Roche), using specific polyclonal rabbit antibodies.

Microscopical techniques
Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described (Waterham et al., 1994). Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific polyclonal antibodies against various peroxisomal proteins or GFP, and gold-conjugated goat-anti-rabbit or goat-anti-mouse antibodies (Waterham et al., 1994). Fluorescence microscopy was performed as described (Baerends et al., 2000).

Results
Reintroduction of Pex3p leads to rapid formation of peroxisomes in pex3 cells
We studied the kinetics of peroxisome re-assembly in pex3 cells of H. polymorpha, upon reintroduction of Pex3p or a Pex3p-GFP fusion by fluorescence and electron microscopical methods. To this end pex3 strains were constructed that contained a single copy of either the PEX3 gene, or a PEX3-GFP fusion under control of the inducible alcohol oxidase promoter integrated in the genome (pex3::P_{AOX} PEX3, and pex3::P_{AOX} PEX3-GFP, respectively) (Baerends et al., 1997). Growth experiments indicated that cells of both transformants had regained the capacity to grow on methanol (data not shown). We analyzed the initial stages of adaptation of the cells from glucose (in which the P_{AOX} is fully repressed) to methanol, which induced the P_{AOX} and thus Pex3p or Pex3p-GFP synthesis. Fluorescence microscopy showed that pex3::P_{AOX} PEX3-GFP cells grown on glucose displayed no fluorescence, as expected (not shown). However, within one hour of culturing on methanol in each cell a single, strong fluorescent spot appeared (see Fig.1). These spots increased in size with time and subsequently multiplied (not shown) to result in the normal WT
Re-assembly of peroxisomes in \( \text{pex3} \) cells

Fig. 1: Fluorescence microscopy of reintroduction of Pex3p-GFP in \( \text{\Delta pex3} \) cells. A single fluorescent spot is observed one hour after the onset of expression of PEX3-GFP by methanol. Bar represents 1 \( \mu \)m.

Fig. 2: Electron microscopical analysis of reintroduction of Pex3p in \( \text{\Delta pex3} \) cells. A: Morphology of strain \( \text{pex3}:P_{\text{AOX}} \text{PEX3} \) grown on glucose. Peroxisomes are lacking completely. B: \( \text{pex3}:P_{\text{AOX}} \text{PEX3} \) cells 1 hour after shift to methanol-containing medium. A single, small peroxisome is present in the vicinity of the nuclear membrane. C, D: Immunocytochemical analysis of \( \text{pex3}:P_{\text{AOX}} \text{PEX3} \) cells after 1 hour of growth in methanol-containing medium using \( \alpha \)-AOX antibodies. The matrix of the newly formed peroxisomes is specifically labeled. Arrow indicates peroxisome, double-headed arrow indicates nuclear membrane. M, mitochondrion, N, nucleus, P, peroxisome. Bars represent 0.5 \( \mu \)m.

phenotype of cells containing several spots (Baerends et al., 2000). These
observations were confirmed by electron microscopical data, shown in Figure 2, which revealed that in all cells a small peroxisome could be observed within 30 minutes after the shift to methanol. Remarkably, the cells generally contained only one peroxisome that increased in size during further cultivation. This organelle was invariably observed in close proximity of the nuclear envelope. Immunocytochemistry showed that these structures contain the peroxisomal membrane protein Pex3p, as well as the matrix protein AO, and therefore indeed represent developing peroxisomes (Fig. 2). These analyses also demonstrated that peroxisome re-introduction in both pex3::P_{AOX}PEX3, and pex3::P_{AOX}PEX3-GFP cells proceeded identically.

Reintroduction of Pex3p in pex3 cells, that artificially produce ER-resident GFP, leads to reassembly of peroxisomes that contain GFP

In order to enable microscopical and biochemical distinction of ER-type membranes from other subcellular compartments, we fused the N-terminal 30 amino acids from the S. cerevisiae ER-located Hsp70 protein BiP to GFP. We showed before that this portion of BiP is sufficient to sort reporter proteins to the ER of H. polymorpha (van der Heide et al., 2002). The constructed strain, pex3::P_{AOX}PEX3::P_{AMO} BiP_{[1-30]}GFP, was grown on glucose/ethylamine-containing media. Under these conditions the alcohol oxidase promoter (P_{AOX}) is fully repressed (by glucose), wereas the amine oxidase promoter (P_{AMO}) is induced by the amine nitrogen source. Fluorescence microscopy, using cells from the mid-exponential growth phase on glucose/ethylamine, showed distinct fluorescence of the nuclear envelope and the peripheral ER (see Figure 3). Subsequently, this strain was transferred to conditions

Fig. 3: Fluorescence microscopy of pex3::P_{AOX}PEX3::P_{AMO} BiP_{[1-30]}GFP. Fluorescence can be observed in the nuclear membrane, as well as in peripheral ER (indicated by arrows). Some cells also show fluorescence inside the vacuole.
that induce peroxisome biogenesis. To this purpose *H. polymorpha* pex3::P_{AOX}PEX3::P_{AMO} BiP\[1-30\]GFP cells were grown to the mid-exponential logarithmic growth phase on glucose/ethylamine until OD\_663 = 1.5, harvested by centrifugation and subsequently resuspended for 30 min in fresh glucose/ammoniumsulphate media at 37 °C, conditions that were previously established to fully deplete P\_AMO-induced mRNAs (Waterham et al., 1993). Next, the cells were transferred to fresh methanol/ammonium sulphate containing media to induce the WT *PEX3* gene, thereby reintroducing functional Pex3p under conditions that fully repress BiP\[1-30\]GFP synthesis (by ammonium ions).

Western blot analysis of crude extracts prepared from these cells shows that during adaptation of the cells to the new methanol environment, both AO and Pex3p are induced, as expected (Fig. 4). Two hours after the shift of cells to methanol, distinct levels of both proteins can be readily detected. The level of GFP, which is present at high levels at the time of the shift (designated T\_0), is gradually decreasing during prolonged growth of cells on methanol and decreased to approximately 10% of the initial level after 12 h of incubation.

Immunocytochemistry revealed that 8 hours after the shift of cells to methanol, peroxisomes were present which contained Pex3p and the peroxisomal matrix protein AO (see Fig. 2). Significant labeling of peroxisomes using GFP-specific antibodies was not observed in immunocytochemical experiments, indicating that GFP levels were below the detection limit (not shown). Also, fluorescence microscopy could not resolve the initially developing peroxisomes in the strong fluorescence background of the nuclear envelope/ER-borne GFP.

Subcellular fractionation of lysates of pex3::P_{AOX}PEX3::P_{AMO} BiP\[1-30\]GFP cells prior to Pex3p reintroduction (T\_0) shows that Pex3p and AO protein are absent in all gradient fractions, as expected (see Figure 5). BiP\[1-30\]GFP largely sedimented to a protein peak in fraction 14 (42 % sucrose), close to the ER membrane marker Sec63p. The
cytosolic marker protein alcohol dehydrogenase (ADH) is found at the top of the gradient. The activity of cytochrome c oxidase, the mitochondrial marker enzyme, is mainly present in fractions 11-16 (46-40 % sucrose). Eight hours after incubation of cells in methanol/ammonium sulphate containing media (T₈), Pex3p and AO are readily detectable in the sucrose density gradient. Both proteins are detected throughout a large part of the gradient (fractions 4-20 for AO, 5-15 for Pex3p) in conjunction with a minor peak of both proteins at fraction 5 (55% sucrose). This corresponds to the expected position of WT *H. polymorpa* peroxisomes (van der Klei et al., 1998). This observation suggests that minor portions of the newly synthesised peroxisomal proteins AO and Pex3p reside in structures that display characteristics of normal WT peroxisomes. However, significant quantities of both proteins are found in fractions with lower density, possibly indicating the presence of these proteins in structures of lower density or leakage (in case of AO). The bulk of BiP₁₋₃₀-GFP largely co-localizes with Sec63p, as is the case at T₀. However, a minor but significant portion of GFP can be detected in higher density fractions, co-localizing with AO and Pex3p. This cannot be due to the presence of contaminating cell membrane vesicles or (fragmented) protoplasts that carry cytoplasmic components as is indicated by the distribution of the cytosolic marker protein ADH. Therefore, these data suggest that a minor amount of BiP₁₋₃₀-GFP is now present in peroxisomes. The controls, ADH and cytochrome c oxidase, sediment in patterns that are largely superimposable on those found at T₀.

The results of the fluorescence and electron microscopical analyses described above already indicate that only a very small fraction of BiP₁₋₃₀-GFP can be detected in the newly formed peroxisomes. Taken together, our data lend support to the notion that the nuclear envelope can act as template for the re-introduction of peroxisomes in *pex3::PₐoX PEX3::PₐmO BiP₁₋₃₀*GFP cells.
**Fig. 5.** Biochemical analysis of reintroduction of Pex3p in Δpex3 cells.

**A, B:** Sucrose density gradients of H. polymorpha Δpex3::PAOX::PEX3::PAMO BIP[1-30]GFP cells grown on glucose/ethylamine. 
**C, D:** Sucrose density gradients of H. polymorpha Δpex3::PAOX::PEX3::PAMO BIP[1-30]GFP pregrown on glucose/ethylamine, eight hours post-shift to methanol/ammonium. 

**A, C:** +: sucrose percentage; ▽: protein mg ml⁻¹; ●: cytochrome C oxidase U ml⁻¹. 

**B, D:** Western blots are shown for: AOX: alcohol oxidase; GFP: BIP[1-30]GFP; ADH: alcohol dehydrogenase; Sec63p; Pex3p as indicated. For detection of Sec63p and ADH, antibodies raised against the Saccharomyces cerevisiae homologues, which cross-react with the corresponding H. polymorpha proteins, were used.
Discussion
In this paper we provide evidence for a role of the endomembrane system in the rescue of peroxisomes in *H. polymorpha* *pex3* cells. *pex3* cells lack morphologically detectable peroxisomal membrane remnants ("ghosts") and thus, hypothetically, a peroxisomal membrane template for peroxisome re-assembly. However, re-introduction of WT Pex3p in the mutant led to the rapid reappearance of a small peroxisome per cell that was invariably localised in close proximity to the nuclear envelope. GFP, accumulated in the ER lumen, including the nuclear envelope, of *pex3* cells appeared to be present in the initial peroxisomes in the complemented cells, suggesting that these membranes served as template for the formation of the organelles.

Previous work in our laboratory has already suggested a putative role of the endomembrane system in one specific case of peroxisome biogenesis. We showed that synthesis of the first 50 amino acids of Pex3p (Pex3p[1-50]) resulted in the formation of vesicles that arose from the nuclear envelope (Faber et al., 2002, chapter 4). These vesicles had the potential to develop into normal peroxisomes upon reintroduction of full-length Pex3p. This implies that mature Pex3p - eventually in conjunction with other peroxisomal membrane proteins (PMPs)- can accumulate all components necessary to develop the vesicles into normal peroxisomes and thus, provide indirect evidence that the nuclear envelope can generate the template for peroxisome re-introduction. Our present data link to and extend these findings to more direct line of evidence that the nuclear envelope indeed can serve as template to allow peroxisome rescue in *H. polymorpha* *pex3* cells.

In *Yarrowia lipolytica* several observations were made that point to an ER - peroxisome assembly relationship. In this organism N-linked core glycosylation of the peroxins Pex2p and Pex16p was observed. This finding suggests that these peroxins have been in contact with the ER lumen during some stage of their presence in the cell (Titorenko and Rachubinski, 1998). Further evidence for a role of the ER in peroxisome biogenesis in this organism came from the observation that the *Y. lipolytica* mutants *sec238* and *srp54*, which are specifically affected in the general secretion route via the ER, are also disturbed in peroxisome biogenesis. Moreover, they accumulate Pex2p and Pex16p in the ER (Titorenko and Rachubinski, 1998). In the same paper, Titorenko *et al.* provide evidence for a multi-step process for peroxisome biogenesis, involving the development of five peroxisomal sub-forms with different characteristics that develop into mature peroxisomes. However, other studies failed to provide evidence for a role for the ER in peroxisome biogenesis in...
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yeast and human cells (South et al., 2000; South et al., 2001; Voorn-Brouwer et al., 2001).

Rescue of peroxisomes in pex mutant cells that lack ghosts has been observed in several organisms. Previous studies on S. cerevisiae, H. polymorpha, Pichia pastoris, Homo sapiens, and Rattus norvegicus pex3 (Höhfeld et al., 1991; Höhfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 1996; Shimozawa et al., 2000; Muntau et al., 2000; Ghaedi et al., 2000), Y. lipolytica and H. sapiens pex16 (Eitzen et al., 1997; Honsho et al., 1998), and S. cerevisiae and H. sapiens pex19 (Hettema et al., 2000; Matsuzono et al., 1999) revealed that peroxisome biogenesis was restored in these mutants upon reintroduction of the corresponding genes. All three pex phenotypes are characterised by the absence of detectable ghosts. The absence of peroxisomal membranes in cells lacking Pex19p has been questioned by the results of Snyder et al. in P. pastoris and Lambkin and Rachubinski (2001) in Y. lipolytica. In P. pastoris pex19 cells small, Pex3p-containing structures have been identified that appear to be different from the ghosts, observed in other pex mutants (Snyder et al., 1999) whereas in Y. lipolytica structures were observed that resemble normal peroxisomes (Lambkin and Rachubinski, 2001). The origin of the newly synthesised peroxisomes is not revealed in detail in any of these studies. In their careful study on the rescue of peroxisomes in a cell line from a Zellweger syndrome patient (PBD061), defective in PEX16, upon introduction of the PEX16 expression vector, Gould and co-workers observed the first new peroxisomal structures in a time span of three hours. On the basis of their data these workers proposed a model for peroxisome rescue in complemented PBD061 cells. This model predicts that Pex16p creates nascent peroxisomes from a yet unidentified structure, termed pre-peroxisome. The nascent peroxisomes subsequently can develop into normal peroxisomes by the import of other PMPs, also including the proliferation factor Pex11p. This is an attractive hypothesis that may also explain how the vesicles that are induced by the synthesis of the first 50 amino acids of Pex3p (Pex3p[1-50]) in H. polymorpha pex3 cells can develop into normal peroxisomes upon synthesis of full-length Pex3p (Faber et al., 2002). Given the fact that Pex3p[1-50] can generate such vesicles, we speculate that the formation of pre-peroxisomes that are predicted in the model of Gould et al., in fact is dependent on Pex3p function. In this view the data of Gould on peroxisome rescue in PBD061 are fully in line with our results in H. polymorpha pex3 cells. Upon Pex3p synthesis, pre-peroxisomal structures are formed that by the incorporation of other PMPs can develop into normal peroxisomes. However, the biochemical properties of the putative pre-peroxisomes structures are still an enigma. Also, the order of events, e.g. an eventual order of
successive incorporation of PMPs in the pre-peroxisomal structure, if any, is fully unknown. Since initially only a single peroxisome is formed per cell, it is difficult to envisage that peroxisome re-assembly in *H. polymorpha pex3* cells follows a similar pathway as described for the multi-step peroxisome development in *Y. lipolytica* (Titorenko et al., 2000). Also, we have to take into account that the data on *Y. lipolytica* cannot be extrapolated to *H. polymorpha* because the principles of peroxisome biogenesis intrinsically differ between the two organisms. Nevertheless, comparative studies are required to solve this point. The putative Pex3p-dependent formation of pre-peroxisomes may also explain why we failed to demonstrate a clear-cut GFP fluorescence in the newly formed peroxisomes in *pex3::P_{AOX}P_{EX3}::P_{AMO}BiP_{[1-30]}GFP* cells since it can readily be envisaged that initially formed organelles are very small. Probably, these structures originate at specialised regions of the nuclear envelope (Faber et al., 2002), which may add to an explanation as to why Gould et al. did not observe any biochemical relation between ER functions and peroxisome biogenesis (South et al., 2001).

The bulk-flow hypothesis for soluble ER protein (Wieland et al., 1987) predicts that the ER/nuclear envelope lumen and the vesicles (initial or pre-peroxisomes) derived from it, contain equal concentrations of GFP. After re-introduction of Pex3p, these initial structures rapidly increase in size, this way diluting the original low amount of GFP throughout the expanding volume of peroxisomal matrix, still allowing GFP demonstration by biochemical but not by fluorescent means. Confocal Laser Scanning Microscopy, a technique that allows analysis of stacks of subsequent sections of biological samples containing fluorescent markers, may represent a promising tool to visualize the early events in future studies on the formation of the GFP-containing pre-peroxisomes.

It is relevant to mention here that we speculate that the above mechanism of peroxisome rescue is not a common mechanism in normally induced WT cells. In such cells peroxisome proliferation proceeds via fission of existing organelles. Most likely the rescue mechanism becomes operative in cells that have lost the organelle, for instance due to a failure in inheritance.

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Chapter 5

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