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Reassembly of peroxisomes in *Hansenula polymorpha pex3* cells on reintroduction of Pex3p involves the nuclear envelope

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nuclear envelope; peroxisome biogenesis;
Pex3p; yeast; green fluorescent protein (GFP).

Abstract

The reassembly of peroxisomes in *Hansenula polymorpha pex3* cells on reintroduction of Pex3p was examined. Using a Pex3-green fluorescent protein (Pex3-GFP) fusion protein, expressed under the control of an inducible promoter, it was observed that, initially on induction of Pex3-GFP synthesis, GFP fluorescence was localized to the endoplasmic reticulum and the nuclear envelope. Subsequently, a single organelle developed per cell that increased in size and multiplied by division. At these stages, GFP fluorescence was confined to peroxisomes. Fractionation experiments on homogenates of *pex3* cells, in which the endoplasmic reticulum and nuclear envelope were marked with GFP, identified a small amount of GFP in peroxisomes present in the initial stage of peroxisome reassembly. Our data suggest a crucial role for the endoplasmic reticulum/nuclear envelope in peroxisome reintroduction on complementation of *pex3* cells by the *PEX3* gene.

Introduction

Peroxisomes are multifunctional organelles, which play an important role in cellular metabolism (for a review, see Purdue & Lazarow, 2001). Several peroxisome-associated disorders have been identified in humans, some of which are lethal (e.g. Zellweger syndrome; reviewed in Weller *et al.*, 2003; Wanders, 2004). In yeasts, peroxisomes are generally involved in the primary metabolism of specific carbon and/or nitrogen sources used for growth.

We have isolated peroxisome-deficient (*pex*) mutants of the methylotrophic yeast *Hansenula polymorpha* and cloned 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* cells (Götte *et al.*, 1998; Honsho *et al.*, 1998; Hettema *et al.*, 2000). All other *pex* mutants identified thus far contain ghosts.

Although ghosts are missing in the *H. polymorpha pex3* mutant, reintroduction and expression of the *PEX3* gene results in the rapid reintroduction of peroxisomes (Baerends *et al.*, 1996). The origin of the membrane of the newly formed

organelles is unknown. The classical model of peroxisome biogenesis involves the growth and multiplication of existing organelles by fission, which implies that new peroxisomes develop from pre-existing ones (Lazarow & Fujiki, 1985). However, recent data support the possibility of alternative pathways for peroxisome biogenesis (for a review, see Titorenko & Rachubinski, 2001; Tabak *et al.*, 2003b).

In this paper, we show that the reintroduction of peroxisomes in *H. polymorpha pex3* cells on expression of the *PEX3* gene proceeds rapidly and involves the endoplasmic reticulum (ER).

Materials and methods

Microorganisms and growth conditions

Hansenula 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. *Escherichia coli* DH5 α (*supE44lacU169* (ϕ 80*lacZ* Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) (Sambrook *et al.*, 1989) was used for recombinant DNA procedures and was

grown on Luria–Bertani (LB) medium supplemented with the appropriate antibiotics.

DNA procedures

Transformation by electroporation of *H. polymorpha* strains and site-specific integration of a single copy of plasmid DNA in the genomic alcohol oxidase (AOX), amine oxidase (AMO) or *PEX14* locus were performed as described previously (Faber *et al.*, 1994a, b; Otzen *et al.*, 2004; Wanders, 2004). Southern blotting was performed using the ECL direct nucleic acid labelling and detection system, as described by the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK). Recombinant DNA manipulations were performed essentially as described previously (Sambrook *et al.*, 1989). Biochemicals were obtained from Roche (Almere, the Netherlands). pHIPZ5-BiP_[1–30]GFP was constructed as follows. The 1.8 kb *SmaI/SpeI* fragment of plasmid pFEM76 (Faber *et al.*, 2002) was cloned into the 4.3 kb *Sall*(Klenow-treated)/*SpeI* fragment of pHIPZ6-Nia. Expression plasmids pHIPX4-PEX3 (Kiel *et al.*, 1995) and pHIPX4-PEX3_[1–50]GFP (pFEM75) (Baerends *et al.*, 2000) have been detailed previously.

Strain constructions

The *H. polymorpha pex3::P_{AOX}PEX3::P_{AMO} BiP_[1–30]GFP* strain, which can be used to independently express the *PEX3* and *BiP_[1–30]GFP* open reading frames in a *pex3* mutant background, was constructed as follows. *NarI*-linearized pZ5-BiP_[1–30]GFP was used to transform *H. polymorpha pex3::P_{AOX}PEX3* (Baerends *et al.*, 1997).

Hansenula polymorpha pex3::P_{AOX}PEX3.GFP was constructed by integrating *SphI*-linearized pFEM152 (*P_{AOX}PEX3.GFP*) (Faber *et al.*, 2001) into the genome of *H. polymorpha* RBG1 strain (*leu1.1Δpex3*) (Baerends *et al.*, 2000).

The *H. polymorpha pex3::P_{AOX}DsRed.SKL::P_{AMO}PEX3.GFP* strain, which can be used to independently express the *PEX3.GFP* and *DsRed.SKL* open reading frames in a *pex3* mutant background, was constructed as follows. Initially, full-length *PEX3* fused to *eGFP* was cloned behind the *P_{AMO}* by cloning a 3.15 kb *Asp718–NcoI* fragment of pFEM152 (Faber *et al.*, 2001) into a 5.5 kb *Asp718–NcoI* fragment of pHIPX5-PEX3 (Kiel *et al.*, 1995), resulting in pHOR45. To obtain a stable strain expressing *PEX3–GFP* behind the *P_{AMO}*, *H. polymorpha* RBG1 (*leu1.1Δpex3*) cells were transformed with *AocI*-linearized pHOR45. A correct single copy integrant was selected by Southern blot analysis for further analysis, and designated RBG43. Finally, *SphI*-linearized pHIPZ4-DsRed-T1.SKL (Monastyrska *et al.*, 2005) was used to transform *H. polymorpha* RBG43.

The *H. polymorpha pex3::PEX14::P_{PEX14}PEX14.GFP* strain, in which the endogenous *PEX14* gene was replaced

by *PEX14.GFP*, was constructed by integrating *Sall*-linearized pHIPX10-PEX14eGFPΔPstI (Otzen *et al.*, 2004) into the genome of *H. polymorpha* RBG1 strain (*leu1.1 pex3*) (Baerends *et al.*, 1996).

Biochemical methods

Cell fractionation experiments (Van der Klei *et al.*, 1998) and the determination of cytochrome c oxidase activities (Douma *et al.*, 1985) were determined as described previously.

Protein concentrations were determined using the BioRad protein assay kit (BioRad GmbH, Munich, Germany), with bovine serum albumin as standard. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out by established procedures. Blots were decorated using the chromogenic (nitroblue tetrazolium-5-bromo-4-chloroindol-3-yl phosphate, NBT-BCIP) or chemiluminescent peroxidase (POD) western blotting kit (Roche), with specific polyclonal rabbit antibodies.

Microscopic techniques

Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously (Waterham *et al.*, 1994). Immunolabelling was performed on ultrathin sections of unicryl-embedded cells, using specific polyclonal antibodies against various peroxisomal proteins or green fluorescent protein (GFP), and gold-conjugated goat antirabbit or goat antimouse antibodies (Waterham *et al.*, 1994). Fluorescence images were obtained using a Zeiss Axioskop fluorescence microscope or a Zeiss LSM510 CLSM (Zeiss Netherlands b.v., Weesp, the Netherlands), as described previously (Otzen *et al.*, 2004).

Results

Reintroduction of Pex3p leads to rapid formation of peroxisomes in *pex3* cells

The kinetics of peroxisome reassembly in *pex3* cells of *Hansenula polymorpha* on reintroduction of Pex3p or a Pex3-GFP fusion protein were studied by fluorescence and electron microscopic 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 AOX 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 analysed the initial stages of adaptation of the cells from glucose (in which *P_{AOX}* is fully repressed) to methanol, which induced *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 1 h of cultivation 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 wild-type (WT) phenotype of cells containing several spots. These observations were confirmed by electron microscopic data (Fig. 2), which revealed that, in all cells, a small peroxisome could be observed within 30 min 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 to the nuclear envelope. Immunocytochemistry showed that these structures contained the peroxisomal membrane protein Pex3p (not shown), as well as the matrix protein AOX (Fig. 2), and therefore indeed represented developing peroxisomes. These analyses also demonstrated that peroxisome reintroduction in both *pex3::P_{AOX}PEX3* and *pex3::P_{AOX}PEX3-GFP* cells proceeded in an identical manner (data not shown).

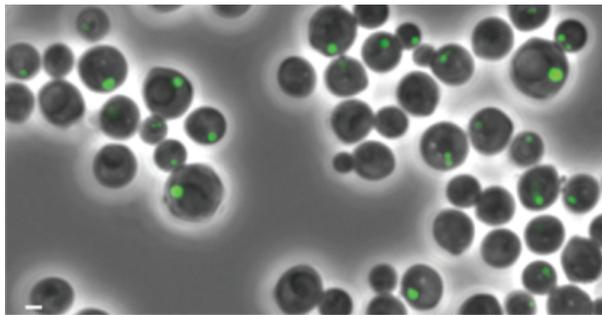


Fig. 1. Fluorescence microscopy of re-introduction of Pex3p-green fluorescent protein (Pex3p-GFP) in *pex3* cells. A single fluorescent spot is observed 1 h after the onset of expression of *PEX3-GFP* induced by methanol. Bar represents 1 μ m.

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

To enable microscopic and biochemical distinction of the ER/nuclear envelope from other subcellular compartments, we constructed a hybrid gene encoding a fusion protein of the N-terminal 30 amino acids from the *Saccharomyces cerevisiae* ER-located Hsp70 protein BiP and GFP. We have shown previously 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 in glucose-ethylamine-containing medium. Under these conditions, the AOX promoter (*P_{AOX}*) is fully repressed (by glucose), whereas the AMO 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 (Fig. 3). Subsequently, this strain was transferred to conditions that induced peroxisome biogenesis. To this end, *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-ammonium sulphate medium 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 medium to induce the WT *PEX3* gene, thereby reintroducing full-length Pex3p under conditions that fully repress *BiP_{[1-30]}}GFP* synthesis (by ammonium sulphate).

Western blot analysis of crude extracts prepared from these cells showed that, during adaptation of the cells to the new methanol environment, both AOX and Pex3p were induced, as expected (Fig. 4). Two hours after the shift of

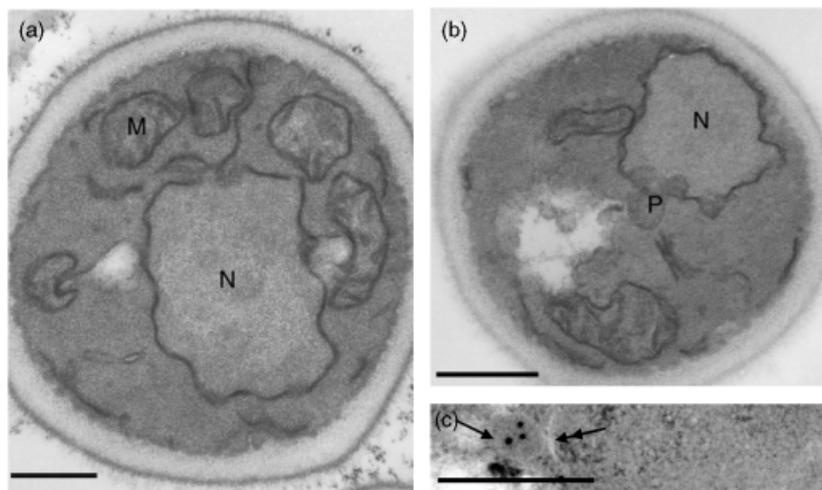


Fig. 2. Electron microscopic analysis of re-introduction of Pex3p in *pex3* cells. (a) Morphology of strain *pex3::P_{AOX}PEX3* grown on glucose. Peroxisomes are lacking completely. (b) *pex3::P_{AOX}PEX3* cells 1 h after shift to methanol-containing medium. A single small peroxisome is present in the vicinity of the nuclear envelope. (c) Immunocytochemical analysis of *pex3::P_{AOX}PEX3* cells after 1 h of growth in methanol-containing medium using anti-alcohol oxidase antibodies. The matrix of the newly formed peroxisomes is specifically labelled. The arrow indicates peroxisome, double-headed arrow indicates nuclear envelope. M, mitochondrion; N, nucleus; P, peroxisome. Bars represent 0.5 μ m.

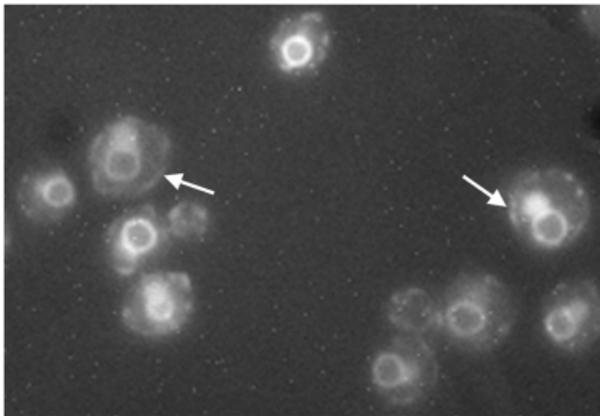


Fig. 3. Fluorescence microscopy of *pex3::P_{AOX}PEX3::P_{AMO} BiP_[1-30]GFP*. Fluorescence can be observed in the nuclear membrane, as well as in the peripheral endoplasmic reticulum (ER) (indicated by arrows). Some cells also show fluorescence inside the vacuole.

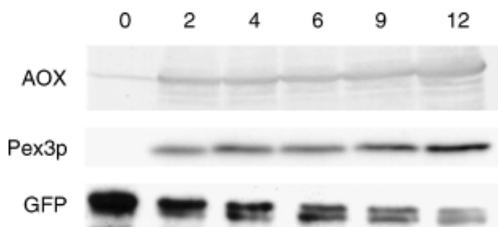


Fig. 4. Protein levels in *pex3::P_{AOX}PEX3::P_{AMO} BiP_[1-30]GFP* cells during adaptation on methanol–ammonium sulphate medium. Crude extracts of cells harvested at indicated time points (indicated in hours) were subjected to sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting. AOX, alcohol oxidase; GFP, BiP_[1-30]GFP.

cells to methanol, distinct levels of both proteins could be readily detected. As expected, the level of GFP, which was present at high levels at the time of the shift ($T=0$ h), gradually decreased during prolonged growth of cells on methanol, and decreased to approximately 10% of the initial level after 12 h of incubation.

Immunocytochemistry revealed that, 1 h after the shift of cells to methanol, peroxisomes were present which contained Pex3p and the peroxisomal matrix protein AOX (see Fig. 2). Significant labelling of peroxisomes using GFP-specific antibodies was not observed in immunocytochemical experiments, indicating that GFP levels were below the detection limit of this method (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 ($T=0$ h) revealed that Pex3p and AOX protein were absent (only shown for AOX) in all gradient fractions, as expected (Fig. 5). BiP_[1-30]GFP largely sedimented to a protein peak

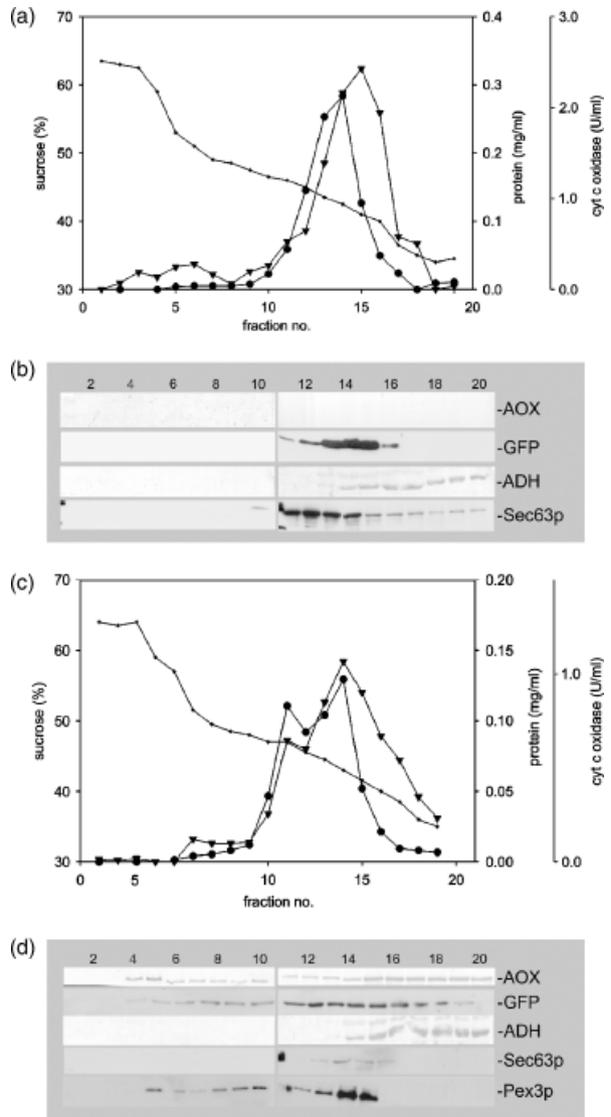


Fig. 5. Biochemical analysis of reintroduction of Pex3p in *pex3* cells. Sucrose density gradients of *Hansenula polymorpha* *pex3::P_{AOX}PEX3::P_{AMO} BiP_[1-30]GFP* cells grown on glucose–ethylamine (a, b) or pregrown on glucose–ethylamine and shifted for 8 h to methanol–ammonium sulphate medium (c, d). (a, c) +, sucrose percentage; \blacktriangledown , protein (mg mL^{-1}); \bullet , cytochrome c oxidase (U mL^{-1}). (b, d) western blots are shown for alcohol oxidase (AOX), BiP_[1-30]GFP (GFP), alcohol dehydrogenase (ADH), Sec63p and Pex3p, as indicated.

in fractions 12–14, similar to the ER membrane marker Sec63p. The cytosolic marker protein aldehyde dehydrogenase (ADH) was detected at the top of the gradient. The activity of cytochrome c oxidase, the mitochondrial marker enzyme, was mainly present in fractions 11–16 (46–40% sucrose). Eight hours after incubation of cells in methanol–ammonium sulphate-containing medium ($T=8$ h), Pex3p and AOX were readily detectable in the sucrose density gradient. Both proteins were detected throughout a large

part of the gradient (fractions 4–20 for AOX, 5–15 for Pex3p), in conjunction with a minor peak of both proteins at fraction 5 (55% sucrose). This position (fraction 5) corresponded to the expected position of WT *H. polymorpha* peroxisomes (Van der Klei *et al.*, 1998). This observation suggests that minor portions of the newly synthesized peroxisomal proteins AOX and Pex3p reside in structures that display biochemical characteristics of normal WT peroxisomes. However, significant quantities of both proteins were found in fractions with lower density, possibly indicating the presence of these proteins in structures of lower density or leakage (in the case of AOX). The bulk of BiP_[1–30]GFP colocalized with Sec63p, as was the case at $T=0$ h. However, a minor but significant portion of GFP was detected in higher density fractions (fractions 5–10), colocalizing with AOX and Pex3p, but not with the ER marker protein Sec63p. This was not due to the presence of contaminating cell membrane vesicles or (fragmented) protoplasts that carry cytoplasmic components, as indicated by the distribution of the cytosolic marker protein ADH. Therefore, these data suggest that a minor amount of BiP_[1–30]GFP was present in peroxisomes. The controls ADH and cytochrome c oxidase sedimented in patterns that were largely superimposable on those found at $T=0$ h.

The results of the fluorescence and electron microscopic analyses described above indicated that only a very small fraction of BiP_[1–30]GFP could be detected in the newly formed peroxisomes. Taken together, our data were consistent with the view that the nuclear envelope could act as a template for the reintroduction of peroxisomes in *pex3::P_{AOX}PEX3::P_{AMO} BiP_[1–30]GFP* cells.

Fluorescence microscopy

Reintroduction of peroxisomes was also followed by fluorescence microscopy. To this end, a strain was constructed that produced Pex3p-GFP under the control of the methyamine-inducible AMO promoter (P_{AMO}), as well as the red fluorescent protein DsRed containing the PTS1 sequence-SKL at its C-terminus (DsRed-SKL) under control of the methanol-inducible P_{AOX} . Cells of this strain were precultivated at conditions that fully repressed the synthesis of both Pex3p-GFP and DsRed-SKL (glucose–ammonium sulphate), followed by the induction of both proteins by shifting the cells to methanol–methylamine-containing medium. In cells grown on glucose–ammonium sulphate to the mid-exponential growth phase, GFP and DsRed fluorescence were not detectable (not shown). After a shift of such cells to methanol–methylamine, the initial GFP fluorescence was localized to distinct structures that, on the basis of their morphology, were likely to represent the nucleus and connecting ER (Fig. 6a). DsRed fluorescence was not detectable at this growth stage (Fig. 6a). After prolonged cultivation, a

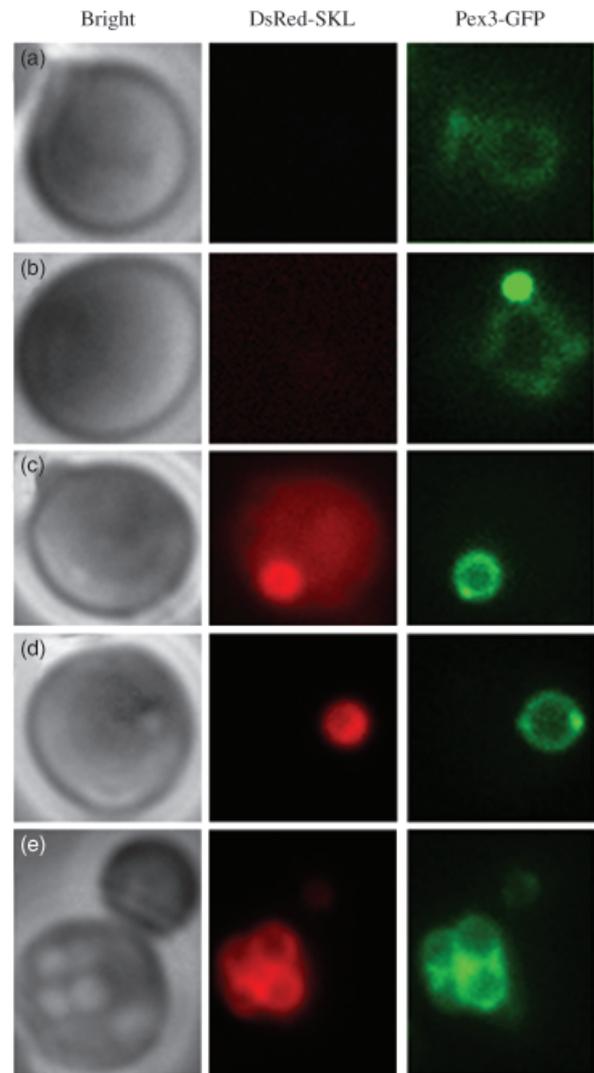


Fig. 6. Fluorescence analysis of peroxisome formation in *Hansenula polymorpha pex3* cells. *Hansenula polymorpha pex3::P_{AOX}DsRed.SKL::P_{AMO}Pex3.GFP* cells were pregrown on glucose–ammonium sulphate and shifted to methanol–methylamine. Initially, green fluorescent protein (GFP) fluorescence was observed, which was localized to structures that, on the basis of their morphology, represented the endoplasmic reticulum (ER)/nuclear envelope (a; 30 min). At later stages, a distinct green fluorescent spot of GFP appeared in these structures (b; 60 min), which, on further induction, developed into round organelles that were no longer attached to the ER/nuclear envelope (c, d; 120 min). These structures represent peroxisomes, as evident from the incorporation of DsRed-SKL, indicated by the presence of red fluorescence (c, d). Finally, the organelles multiplied by division, similar to that observed in normal wild-type (WT) cells (e; 17 h after the shift).

distinct GFP fluorescent spot developed, and weak DsRed fluorescence appeared in the cytosol (Fig. 6b). This spot developed in a round structure that also accumulated DsRed (Fig. 6c), and therefore was considered to represent a developing peroxisome. Inside this peroxisome, GFP

fluorescence was enhanced at the periphery of the organelle, which is normally seen in peroxisomes of methanol-grown *H. polymorpha* cells as a result of the presence of a large AOX crystalloid inside peroxisomes. After further cultivation, all cytosolic red fluorescence accumulated in this structure (Fig. 6d), after which the organelle multiplied (Fig. 6e). Typically, as observed previously (Haan *et al.*, 2002), a portion of Pex3p-GFP accumulated in a focal spot at the peroxisomal membrane (Fig. 6c–e). Taken together, these data were consistent with the view that the new peroxisomes in these cells derived from a subcellular membrane system, most likely the nucleus/ER system.

***Hansenula polymorpha pex3* cells lack peroxisomal membrane structures**

Data obtained in *Pichia pastoris* (Hazra *et al.*, 2002) have suggested that *P. pastoris pex3* cells contain peroxisomal membrane remnants. Despite various biochemical and immunocytochemical studies, however, we were invariably unable to detect such structures in *H. polymorpha pex3* cells (Baerends *et al.*, 1996, 1997; Van der Klei *et al.*, 1998). We re-examined this issue using fluorescence microscopy to analyse the localization of two peroxisomal membrane proteins, Pex10p and Pex14p. To this end, we analysed the localization of Pex10-cyan fluorescent protein (CFP) and Pex14-GFP fusion proteins (both expressed under the control of their own promoters). However, Pex10-CFP fluorescence was below the limit of detection. Moreover, we were unable to detect any Pex10-CFP by Western blot analysis, whereas in WT controls this protein was easily detectable (data not shown). In the case of Pex14-GFP, we observed strong spots of GFP fluorescence that localized to the mitochondria (Fig. 7). This observation indicated that the normal target membrane for Pex14p-GFP was absent in *H. polymorpha pex3* cells. Similar findings (undetectable Pex10p levels and mislocalization of Pex14-GFP to the mitochondria) have been reported previously for *H. polymorpha pex19* cells, which are devoid of peroxisomal membrane remnants (Otzen *et al.*, 2004). Hence, our data suggested that, as in *H. polymorpha pex19* cells, in *H. polymorpha pex3* cells peroxisomal membrane remnants were absent.

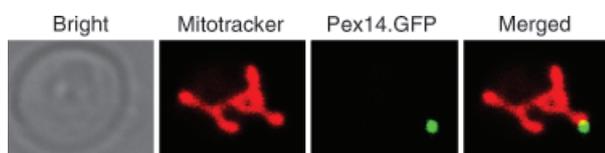


Fig. 7. Localization of Pex14p-green fluorescent protein (Pex14p-GFP) in *Hansenula polymorpha pex3* cells. Analysis by confocal laser scanning microscopy indicated that green fluorescence, due to HpPex14-GFP, was present as a distinct spot on the mitochondria (stained in red by Mitotracker).

Discussion

In this paper, we have provided evidence for a role of the ER/nuclear envelope in the reintroduction of peroxisomes in *Hansenula polymorpha pex3* cells. *pex3* cells lack morphologically detectable peroxisomal membrane remnants ('ghosts') and thus, hypothetically, a peroxisomal membrane template for peroxisome reassembly (Baerends *et al.*, 1996, 1997; Van der Klei *et al.*, 1998) (this study). However, reintroduction of WT Pex3p in the mutant led to the rapid reappearance of one small peroxisome per cell. GFP, accumulated in the ER lumen, including the nuclear envelope, of *pex3* cells appeared to be present in the initial peroxisomes of the complemented cells, suggesting that these membranes served as a template for the formation of the organelles.

Previous work in our laboratory has already suggested a role of the nuclear envelope in one specific case of peroxisome biogenesis. We showed that the synthesis of the first 50 amino acids of Pex3p (Pex3_[1–50]) resulted in the formation of vesicles that arose from the nuclear envelope (Faber *et al.*, 2002). These vesicles had the potential to develop into normal peroxisomes on reintroduction of full-length Pex3p. This implies that mature Pex3p—eventually in conjunction with other peroxisomal membrane proteins—can accumulate all the components necessary to develop the vesicles into normal peroxisomes, providing indirect evidence that the nuclear envelope can generate the template for peroxisome reintroduction. Our present data link and extend these findings to a more direct line of evidence that the ER/nuclear envelope can indeed serve as a template to allow peroxisome rescue in *H. polymorpha pex3* cells.

In *Yarrowia lipolytica*, several observations have been 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 suggested that these peroxins had been in contact with the ER lumen during some stage of their presence in the cell (Titorenko & 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, were also disturbed in peroxisome biogenesis. Moreover, they accumulated Pex2p and Pex16p in the ER (Titorenko & Rachubinski, 1998). In the same paper, these authors provided evidence for a multistep process for peroxisome biogenesis, involving the development of five peroxisomal subforms with different characteristics that developed into mature peroxisomes.

Other data have been presented that point to a role of the ER in peroxisome biogenesis (reviewed in Tabak *et al.*, 2003a). Moreover, using immunocytochemistry and tomography, a continuity was demonstrated between maturing peroxisomes and specialized regions of the ER in mouse

dendritic cells (Geuze *et al.*, 2003). However, several other studies failed to provide evidence for a role of the ER in peroxisome biogenesis (South *et al.*, 2000, 2001; Voorn-Brouwer *et al.*, 2001; Tabak *et al.*, 2003a).

The rescue of peroxisomes in *pex* mutant cells that lack ghosts (e.g. *pex3*, *pex16* and *pex19* mutants) has been observed in several organisms (Purdue & Lazarow, 2001). Moreover, it has been suggested that the ER plays a role in this process (Bascom *et al.*, 2003). However, the absence of peroxisomal membranes in *Pichia pastoris* strains lacking Pex3p (Hazra *et al.*, 2002) or Pex19p (Snyder *et al.*, 1999) has been questioned. Moreover, in *Y. lipolytica pex19* cells, structures were observed that resembled normal peroxisomes (Lambkin & Rachubinski, 2001). In *H. polymorpha pex19* cells, peroxisomes and peroxisomal membrane remnants were fully absent. However, on overproduction of Pex3p in such cells, peroxisomal structures were formed (Otzen *et al.*, 2004). The latter observation strongly suggests that these structures were not formed from pre-existing peroxisomes or ghosts, but from another membrane source (e.g. the ER) (Otzen *et al.*, 2004).

The origin of the newly synthesized peroxisomes has not been revealed in detail in any of these studies (Tabak *et al.*, 2003a). In their careful study on the rescue of peroxisomes in a cell line from a Zellweger syndrome patient (PBD061) defective in *PEX16*, on introduction of the *PEX16* expression vector, South & Gould (1999) observed the first new peroxisomal structures in a time span of 3 h. On the basis of their data, these workers proposed a model for peroxisome rescue in complemented PBD061 cells. This model predicted that Pex16p created nascent peroxisomes from a yet unidentified structure, termed the preperoxisome. The nascent peroxisomes subsequently developed into normal peroxisomes by the import of other peroxisomal membrane proteins, 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 (Pex3_[1–50]) in *H. polymorpha pex3* cells can develop into normal peroxisomes on synthesis of full-length Pex3p (Faber *et al.*, 2002). Given the fact that Pex3_[1–50] can trigger or mediate the formation of such vesicles, we speculate that the formation of preperoxisomes, predicted in the model of South & Gould (1999), is dependent on Pex3p function. In this view, the data of South & Gould (1999) on peroxisome reintroduction in PBD061 cells are fully in line with our results in *H. polymorpha pex3* (this study) and *pex19* (Otzen *et al.*, 2004) cells. On Pex3p synthesis, preperoxisomal structures are formed that, by the incorporation of other peroxisomal membrane proteins, can develop into normal peroxisomes in *pex3* cells. However, the biochemical properties of the putative preperoxisome structures are still unclear. In addition, the order of events, e.g. successive incorporation of peroxisomal membrane proteins in the preperoxisomal

structure, if any, is unknown. As, initially, only a single peroxisome is formed per cell, it is difficult to envisage that peroxisome reassembly in *H. polymorpha pex3* cells follows a similar pathway as described for the multistep peroxisome development in *Y. lipolytica* (Titorenko *et al.*, 2000).

The Pex3p-dependent formation of preperoxisomes may also explain why we failed to demonstrate a clear-cut GFP fluorescence in the newly formed peroxisomes in *pex3::P_{AOX}PEX3::P_{AMO} BiP_[1–30]GFP* cells, as it can readily be envisaged that the initially formed organelles will be very small. These structures probably originate in specialized regions of the nuclear envelope (Faber *et al.*, 2002), which may add to the explanation of why South *et al.* (2001) did not observe any biochemical relation between ER functions and peroxisome biogenesis.

The bulk-flow hypothesis for soluble ER protein (Wieland *et al.*, 1987) predicts that the ER/nuclear envelope lumen, and the vesicles (initial or preperoxisomes) derived from it, will contain equal concentrations of GFP. After reintroduction of Pex3p, these initial structures rapidly increase in size, diluting the original small amount of GFP throughout the expanding volume of the peroxisomal matrix, still allowing GFP demonstration by biochemical but not fluorescence techniques.

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

While this paper was under review, restoration of peroxisome formation was also shown to involve the ER in *Saccharomyces cerevisiae pex3* mutants on expression of *PEX3* (Hoepfner *et al.*, 2005; Kragt *et al.*, 2005; Tam *et al.*, 2005).

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