Chapter 4

Normal peroxisome development from vesicles induced by truncated *Hansenula polymorpha* Pex3p

Klaas Nico Faber, Gert Jan Haan, Richard J.S. Baerends, Anita M. Kram, Marten Veenhuis

This chapter was published in:

Abstract
We show that the synthesis of the N-terminal 50 amino acids of Pex3p \([\text{Pex3p}_{1-50}]\) in *Hansenula polymorpha* *pex3* cells is associated with the formation of vesicular membrane structures. Biochemical and ultrastructural findings suggest that the nuclear membrane is the donor membrane compartment of these vesicles. These structures also contain Pex14p and can develop into functional peroxisomes after subsequent re-introduction of the full-length Pex3p protein. We discuss the significance of this finding in relation to peroxisome re-introduction, e.g. in case peroxisomes are lost due to failure in inheritance.

Introduction
Peroxisomes are remarkable among the various classes of cell organelles in that their function and abundance varies dependent on the organism, the environmental conditions and the physiological state of the cell. Yeasts are favorable model organisms to study peroxisome biogenesis because of a number of properties. Firstly, peroxisome proliferation can be strictly regulated by growth conditions, ranging from one small organelle when cells are grown in rich media containing glucose, to over 20 during growth of cells on oleate (*Saccharomyces cerevisiae, Yarrowia lipolytica* and *Pichia pastoris*) or methanol (*Hansenula polymorpha, P. pastoris*) (Veenhuis, 1992; Kunau, 1998). Under these conditions, peroxisomes house the key enzymes involved in the metabolism of these carbon sources. Secondly, peroxisomes are not essential for yeast cell viability when they are grown under peroxisome-repressing conditions in rich media. This feature has led to the isolation of various yeast peroxisome assembly (*pex*) deficient mutants that are unable to utilise either oleate or methanol for growth (Erdmann et al., 1997; Subramani, 1998; Hettema et al., 1999). With few exceptions (see below), these *pex* mutant cells still contain peroxisomal membrane remnants (“ghosts”). Through genetic complementation, 23 *PEX* genes have currently been characterised (Hettema et al., 1999). Database searches using the yeast genes have presently revealed 13 human orthologues of yeast *PEX* genes. Mutations in 11 of these human *PEX* genes have been characterised and shown to be the molecular basis for inherited peroxisome biogenesis disorders, including Zellweger Syndrome, neonatal adrenoleukodystrophy, infantile Refsum’s disease and rhizomelic chondrodysplasia punctata (Braverman et al., 1995; Wanders, 1999).

The analysis of the *PEX* genes and the peroxins they encode has predominantly shed light on the molecular components involved in peroxisomal matrix protein
Peroxisome development from vesicles

import (reviewed by (Subramani, 1998; Hettema et al., 1999). Relatively less is known of the biogenesis of the peroxisomal membrane and the sorting mechanisms of the proteins it contains. Earlier work showed that peroxisomal membrane proteins, like peroxisomal matrix proteins, are synthesised on free polysomes in the cytosol and are post-translationally transported to the peroxisome (Fujiki et al., 1984). Together with morphological studies on peroxisome proliferation, these data have led to the hypothesis that peroxisomes develop by fission from pre-existing ones (Lazarow and Fujiki, 1985). Recent research, however, suggested that some peroxisomal membrane proteins may travel via the ER to the peroxisome and that vesicle budding and fusion processes may be involved in peroxisome growth and maturation (Titorenko and Rachubinski, 1998; Faber et al., 1998; Mullen et al., 1999; Titorenko et al., 2000).

Notably, the prevailing model of budding from pre-existing peroxisomes cannot explain the re-assembly of peroxisomes in mutant cells lacking any peroxisomal remnants (yeast and human pex3, H. polymorpha per13-6ts, human pex16, and S. cerevisiae and human pex19) upon functional complementation of such cells (Höhfeld et al., 1991; Waterham et al., 1993; Baerends et al., 1996; Wiemer et al., 1996; Götte et al., 1998; Matsuzono et al., 1999; South and Gould, 1999; South et al., 2000). Several recent studies have assessed the question of the origin of the peroxisomal membrane upon re-appearance of the organelles in pex-mutants lacking peroxisomal membranes. We studied this process in a temperature-sensitive (ts) pex mutant of the yeast H. polymorpha (Waterham et al., 1993). Peroxisomal membrane remnants were undetectable in these cells when grown at restrictive temperatures (43°C). Shifting these cells to permissive conditions (37°C) led to a rapid (within 30 min) reappearance of peroxisomes. Concurrently, South and Gould (1999), Matsuzono et al. (1999) and South et al. (2000) studied the reappearance of peroxisomes in human cells defective for PEX16, PEX19 and PEX3, respectively. Peroxisomal remnants were absent in these cells also, but intact organelles were assembled upon re-introduction of the complementing gene. As suggested before by Waterham et al. (1993), these studies revealed that peroxisomes do not necessarily derive from pre-existing ones, but failed to identify the alternative origin.

Therefore, we set out to study the reappearance of peroxisomes in H. polymorpha pex3 cells in detail upon re-introduction of full-length Pex3p, a peroxisomal membrane protein, and hybrid proteins consisting of N-terminal fragments of Pex3p and either GFP or β-lactamase. Here we show that the N-terminal 50 amino acids of Pex3p induce the formation of vesicles in the vicinity of the nuclear membrane.
These Pex3p[1-50] vesicles are the specific target for peroxisome development after subsequent synthesis of full-length Pex3p.

Materials and methods

Strains and growth conditions

_H. polymorpha_ NCYC495 (_leu1.1_) and derivatives of this strain (listed in Table I) were grown at 37°C in batch cultures in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) or in mineral medium (van Dijken et al., 1976) containing either 0.5% (w/v) glucose, 0.5% (v/v) methanol or a mixture of 0.1% (v/v) glycerol and 0.5% methanol as carbon and energy source in combination with 0.25% (w/v) ammonium sulphate or 0.25% (w/v) ethylamine as sole nitrogen sources. For growth on solid media, 0.67% (w/v) yeast nitrogen base was used supplemented with 1% (w/v) glucose, and 2% (w/v) agar. When required, leucine was added to the media to a final concentration of 30 mg/l.

Table I. _H. polymorpha_ strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCYC495</td>
<td><em>H. polymorpha</em> WT, <em>leu1.1</em> derivative</td>
<td>(Gleeson and Sudbery, 1988)</td>
</tr>
<tr>
<td>RBG1</td>
<td>pex3::URA3, <em>leu1.1</em></td>
<td>(Baerends et al., 2000)</td>
</tr>
<tr>
<td>RBG17</td>
<td>pex3::P&lt;sub&gt;AOX&lt;/sub&gt;-PEX3&lt;sup&gt;3x&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>HF75</td>
<td>NCYC495::P&lt;sub&gt;AOX&lt;/sub&gt;-PEX3&lt;sub&gt;[1-50]&lt;/sub&gt;GFP&lt;sup&gt;2x&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>HF78</td>
<td>pex3::P&lt;sub&gt;AOX&lt;/sub&gt;-PEX3&lt;sub&gt;[1-50]&lt;/sub&gt;GFP&lt;sup&gt;3x&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>HF245</td>
<td>pex3::P&lt;sub&gt;AOX&lt;/sub&gt;-PEX3&lt;sub&gt;[1-50]&lt;/sub&gt;β-glucosamine&lt;sup&gt;x&lt;/sup&gt;:P&lt;sub&gt;AMO&lt;/sub&gt;-BiP&lt;sub&gt;[1-30]&lt;/sub&gt;GFP&lt;sup&gt;2x&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>HF290</td>
<td>pex3::P&lt;sub&gt;AOX&lt;/sub&gt;-PEX3&lt;sub&gt;[1-50]&lt;/sub&gt;GFP&lt;sup&gt;3x&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>HF305</td>
<td>pex3::P&lt;sub&gt;PEX2&lt;/sub&gt;-PEX3&lt;sub&gt;[1-50]&lt;/sub&gt;GFP&lt;sup&gt;3x&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

Superscript (1x, 2x or 3x) refers to the number of expression cassettes integrated in the genome of the transformed strain.

Molecular biological techniques

_Escherichia coli_ DH5α and XL1blue were used for the propagation and amplification of plasmid DNA. Recombinant DNA procedures (enzyme digestion, cloning, plasmid isolation, PCR and Southern blotting) were performed essentially as described by (Sambrook et al., 1989). Transformation of _H. polymorpha_ strains and site-specific integration of single and multiple copies of plasmid DNA in the genomic _AOX-_.

76
AMO-locus was performed as described (Faber et al., 1994a; Faber et al., 1994b; Baerends et al., 1996).

**Plasmid constructions**

Expression plasmids pHIPX4-PEX3 (Kiel et al., 1995) and pHIPX4-PEX3[1-50]GFP (pFEM75) (Baerends et al., 2000) have been detailed before. pHIPX6-PEX3[1-50]GFP (pFEM64) was obtained by inserting a 0.9 kb BamHI-Sall from pFEM75 into BamHI-Sall digested pHIPX6 (Kiel et al., 1995), an H. polymorpha expression plasmid containing the PEX3 promoter element. For co-expression of reporter genes, novel H. polymorpha expression vectors were constructed based on the dominant zeocin resistance gene. Vector pHIPZ4, containing the H. polymorpha alcohol oxidase promoter (PAOX) for heterologous expression has recently been described (Salomons et al., 2000). pHIPZ5, containing the H. polymorpha amine oxidase promoter (PAMO) was constructed by inserting an 1.0-kb NotI-BamHI DNA fragment from pHIPX5 (Kiel et al., 1995) containing the PAMO into NotI-BamHI digested pHIPZ4. pHIPZ4-PEX3[1-50]β-lactamase (pFEM201) was constructed as follows: the β-lactamase reporter gene was subcloned in Bluescript II SK⁺ (Stratagene, La Jolla, CA) as a 0.8-kb EcoRI-HindIII fragment from pGF154 (Faber et al., 1995) and subsequently inserted as a Smal-Sall fragment into pBS-PEX3 (Baerends et al., 2000) digested with Ncol (Klenow fill-in)-Sall, resulting in pFEM32. Subsequently, a 0.15-kb PCR fragment obtained using primers per9ATG (Kiel et al., 1995) and pex3-FT2 (Baerends et al., 2000) was digested with BglII and Ncol and inserted in BglII-Ncol digested pFEM32, resulting in pFEM30. The PEX3[1-50]β-lactamase hybrid gene was excised from pFEM30 by BamHI-Sall digestion and inserted into BamHI-Sall digested pHIPZ4, resulting in pFEM201. pHIPZ5-PEX3[1-50]GFP (pFEM167) was constructed as follows: Using PCR and primers KN18 (5' CCCAAGCTT GGATCC ATG TTA ACT TTC AAT AAG TC 3') and KN19 (5' GGGAAAGCTT AGATCT AAA CTG CTG TGT TAG TGG G 3') a BamHI site was introduced upstream the startcodon, and a BglII site downstream codon 30 (Phe) of the H. polymorpha KAR2 gene. In addition, using PCR and primers KN14 (5' CCCCTC GAG AAC CTG TAC TTC CAG TCG AGA TCT GTG TGG G 3') a BamHI site was introduced upstream the startcodon, and a BglII site downstream the stopcodon of the eGFP gene (Clonetech, Germany). The BglII sites were used to fuse the BiP[1-30] and the eGFP genes. The flanking BamHI and the SalI
sites were used to clone the hybrid gene downstream the amine oxidase promoter in expression vector pHIPX5.

**Biochemical methods**

Crude extracts of *H. polymorpha* were prepared according to (Baerends et al., 2000). SDS-PAGE (Laemmli, 1970) and Western blot analysis (Kyhse-Andersen, 1984) were performed as described; blots were decorated using specific antibodies against various *H. polymorpha* proteins. The antibodies against *S. cerevisiae* Sec63p, which cross-react with the *H. polymorpha* Sec63p orthologue, were a gift from Dr. R. Schekman, Berkeley, USA. The antibodies against GFP, and *S. cerevisiae* cytosolic ADH, which cross react with the *H. polymorpha* ADH orthologue, were a gift from Dr. W.-H. Kunau, Bochum, Germany. Goat anti-rabbit alkaline phosphatase and goat anti-rabbit horse radish peroxidase (Roche Molecular Biochemical, Almere, The Netherlands) were used as secondary antibodies which were detected by bromochloroindolyl phosphate/Nitro blue tetrazolium (Roche Molecular Biochemical, Almere, The Netherlands) or ECL (Amersham, Arlington Heights, IL) according to manufacturers’ protocols. Enzyme activity of cytochrome c oxidase was determined as described (Douma et al., 1985). Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad GmbH, Munich, Germany) using bovine serum albumin as standard. Cell fractionation experiments were performed as detailed by van der Klei *et al.* (van der Klei *et al.*, 1998). In addition to the standard sucrose density gradients routinely used to purify peroxisomes from WT *H. polymorpha*, we used a modified gradient (consisting of 4 ml 65%, 4 ml 50%, 4 ml 46%, 8 ml 40% 4 ml 35% and 4 ml 30% (w/v)-sucrose in buffer B (5 mM MES, 0.1 mM EDTA, 1 mM KCl, pH 5.5) loaded with an organellar pellet resuspended in 5 ml homogenisation buffer (5 mM MES, 0.1 mM EDTA, 1 mM KCl, 1.2 M sorbitol, pH 5.5) and centrifuged for 3 h at 33,000 x g.

**Microscopy**

Fluorescent microscopy to localise hybrid proteins containing GFP was carried out according to Baerends *et al.*, (2000).

Whole cells and organellar fractions were fixed and prepared for electron microscopy and immunocytochemistry as described previously (Douma *et al.*, 1987; Waterham *et al.*, 1994). Immunolabelling was performed on ultrathin sections of unicycryl-embedded cells, using specific antibodies against various *H. polymorpha* proteins, GFP and β-lactamase and gold-conjugated goat anti-rabbit (GAR-gold) antibodies
according to the instructions of the manufacturer (Amersham Corp. Arlington Heights, IL). Double immunocytochemical labelling using two polyclonal antisera and different sized gold particles (5-nm and 15-nm GAR-gold) was performed according to Bendayan (1982).

Results

Synthesis of Pex3p\(_{\text{1-50}}\) causes vesicle formation in pex3 cells

Similar to pex3 cells from other organisms, \(H.\ polymorpha\) pex3 cells grown in batch cultures on methanol, do not contain detectable peroxisomal membrane remnants and proteins normally residing in the peroxisomal matrix, accumulate in the cytosol. However, normal peroxisomes rapidly re-appear in such cells upon re-introduction of the complementing \(\textit{PEX3}\) gene (Baerends et al., 1996). In an attempt to shed light on the origin of these organelles, we introduced hybrid genes encoding N-terminal fragments of Pex3p (containing its putative peroxisomal targeting signal) and a reporter gene (GFP or \(\beta\)-lactamase) in pex3 cells and determined the subcellular location of the gene products. pex3 cells producing the Pex3p\(_{\text{1-50}}\)GFP hybrid protein (strain HF78) contained one or few bright fluorescent spots (Fig. 1B right panel); in WT controls (strain HF75) this protein was efficiently targeted to peroxisomes (Fig 1A, right panel; (Baerends et al., 2000). Electron microscopy showed that HF78 cells contained a cluster of small vesicular structures in close proximity to the nucleus (Fig. 1C). These structures were never observed in the pex3 parental strain (not shown, cf. (Baerends et al., 1996) or in WT cells. These membrane structures were the subcellular site of the Pex3p\(_{\text{1-50}}\)GFP hybrid protein as was shown by immunocytochemistry, using \(\alpha\)-GFP antibodies (Fig. 1D). The presence of these membranes was not the result of the overproduction of the hybrid protein. Similar patterns of fluorescence (Fig 1C inset) and membrane vesicles (not shown) were observed when the Pex3p\(_{\text{1-50}}\)GFP protein was produced by the \(\textit{PEX3}\)-promoter element, though less abundantly. These data show that synthesis of the 50 N-terminal amino acids of Pex3p fused to a reporter protein, induces proliferation of membranous structures. From here, we refer to these membrane structures as Pex3p\(_{\text{1-50}}\)-vesicles.
Pex3p[1-50]-vesicles display peroxisomal characteristics.

To determine the nature of the Pex3p[1-50]-vesicles, we set out to purify them, using GFP as a marker protein. The conventional biochemical procedure for the isolation of intact peroxisomes from *H. polymorpha* WT cells [homogenisation of protoplasts followed by sucrose-density gradient centrifugation of a post-nuclear supernatant (PNS)] applied on HF78 cells resulted in a distinct localisation of Pex3p[1-50]GFP at densities of approximately 30-40% sucrose, colocalising with ER and mitochondrial marker proteins (Fig. 2). To separate the Pex3p[1-50]-vesicles from other cell constituents, we adjusted the sucrose density profile of the gradient to obtain a better
Fig. 2. Pex3p[1-50]-vesicles migrate to low-density fractions after sucrose density gradient centrifugation. Sucrose density centrifugation of a post-nuclear supernatant (PNS) obtained from glycerol/methanol-grown HF78 cells. Individual fractions were analysed for sucrose density (+), protein concentration (o) and mitochondrial cytochrome c oxidase activity (△). Western blot analysis was used to determine the location of alcohol oxidase (AOX), Pex3p[1-50]GFP (GFP), mitochondrial porin, endoplasmic reticulum Sec63p and peroxin Pex14p. All membrane-bound marker proteins co-localise in the top fractions of the gradient.

Separation at sucrose concentrations of 30% to 40% and loaded the gradient with an organellar fraction instead of a PNS. Fig. 3 shows that a clear separation was achieved between the Pex3p[1-50]-vesicles (peak fractions at 32-36% sucrose) and the mitochondria (peak fractions at 36-42% sucrose). Comparison of the fractions most enriched for Pex3p[1-50]GFP, mitochondria and ER marker proteins (peak fractions at 38-41% sucrose) revealed that Pex14p, a peroxisomal membrane protein that is mislocalised to mitochondria in pex3 cells (Baerends et al., 2000), now co-localised with Pex3p[1-50]GFP (Fig. 3B). This finding was further substantiated by immunocytochemical analysis of the organellar fraction (Fig. 4A,B) and whole cells (Fig. 6E, see below). The Pex3p[1-50]-vesicles were readily identifiable in the organellar fraction by immunocytochemistry, using specific antibodies against GFP, and were clearly distinct from other structures in these fractions, such as mitochondria and plasma membrane vesicles (Fig. 4A). Clearly, α-Pex14p-dependent specific labelling was only found on the Pex3p[1-50]-vesicles (Fig. 4B). In addition, co-localisation of Pex3p[1-50]β-lactamase and endogenous Pex14p on the Pex3p[1-50]-vesicles was observed in double-labelling experiments performed on ultrathin sections of pex3 cells producing Pex3p[1-50]β-lactamase (Fig. 6E).

To further analyse the possible peroxisomal nature of the Pex3p[1-50]-vesicles, we investigated whether these structures were susceptible to selective degradation, comparable to WT peroxisomes (Veenhuis et al., 1983) and peroxisomal remnants.
(ghosts) in methanol-induced H. polymorpha pex cells (Veenhuis et al., 1996). To this end, HF75 (WT::P_{AOX}-PEX3_{1-50}GFP) and HF78 cells (pex3::P_{AOX}-PEX3_{1-50}GFP) were grown in glycerol/methanol-containing media to induce peroxisome- and Pex3p_{1-50}-vesicle formation, respectively. Next, these cells were transferred to fresh glucose-media, thus repressing peroxisome- and vesicle-formation, and the fate of AO and GFP was followed in time. As expected, in HF75 control cells both the level of peroxisomal AO and Pex3p_{1-50}GFP decreased significantly (Fig. 3C) and after 4 h of induction in glucose medium, few small peroxisomes were observed by fluorescence microscopy and electron microscopy (data not shown). In HF78 cells, however, significant amounts of AO remained detectable at this stage, showing that this cytosolic protein was not actively degraded under these conditions (van der Klei
et al., 1991). In contrast, the level of Pex3p[1-50] GFP decreased with similar kinetics as observed for HF75 cells, suggesting that the Pex3p[1-50] GFP-vesicles were actively degraded after a shift of HF78 cells to glucose-medium.

Taken together, these data suggest that the Pex3p[1-50]-vesicles are not merely a sink for non-functional proteins, but instead represent membrane vesicles that show peroxisomal properties.

Fig. 4. Pex14p is present on Pex3p[1-50]-vesicles. Immunolabelling of the organelar fraction loaded onto the sucrose gradient analysed in Fig. 3A, using antibodies against GFP (A) and Pex14p (B). Typical clusters of small vesicles were specifically labelled using these antibodies. Abbreviations: M, mitochondrion. Bar = 0.2 µm.

Fig. 5. Control of protein synthesis in HF245 cells. A, equal amounts of total protein extracts of HF245 (pex3::P_AOX-PEX3::P_AMO-PEX3[1-50]GFP) cells, grown in media containing glucose/ammonium sulphate (lane 1), glucose/ethyamine (lane 2) or methanol/ammonium sulphate (lane 3), were analysed by Western blotting for the presence of AOX, Pex3p, AMO, Pex3p[1-50]GFP and elongation factor 1α (EF-1α; constitutively synthesised protein used as control). Synthesis of AOX and Pex3p was specifically induced in methanol-containing media, whereas AMO and Pex3p[1-50]GFP were detected only in amine-containing media. B, Western blot analysis of total protein extracts of HF245 (pex3::P_AOX-PEX3::P_AMO-PEX3[1-50]GFP) cells shifted (at t=0) from glucose/ethyamine- to glycerol/methanol/ammonium sulphate medium. One hour after the shift, Pex3p and AOX are detectable, whereas Pex3p[1-50]GFP (GFP) remains detectable even after 24 h after the shift.
Pex3p[1-50]-vesicles develop at the nuclear membrane.

Next, we investigated the origin of the vesicles induced upon production of Pex3p[1-50]GFP. To this end, we constructed a strain, HF245, in which Pex3p[1-50]-vesicle-production and full-length Pex3p synthesis can be separately regulated. This strain is deleted for the endogenous PEX3 gene. Instead, it contains the PEX3 gene under control of the methanol-inducible alcohol oxidase promoter (P_AOX) together with the gene encoding Pex3p[1-50]GFP under control of the amine-inducible amine oxidase promoter (P_AMO) (Fig. 5A). HF245 cells were shifted from P_AMO-repressing conditions (glucose/ammonium sulphate), to media that induce the P_AMO (glucose/ethylamine). Within 1 h after the shift, small fluorescent profiles could be detected in the cells by fluorescence microscopy. After prolonged incubation the brightness of these spots increased and only rarely we observed more than one fluorescent spot per cell (not shown, cf. Fig. 1B). Other fluorescent subcellular structures were not detected by fluorescence microscopy. A detailed electron microscopic analysis of the initial stages of the vesicle proliferation showed that these structures invariably developed in close vicinity of the nuclear membrane (Fig. 6A). Immunocytochemically, Pex3p[1-50]GFP was solely detectable at these membranes (Fig. 6B). We never observed the development of a cluster of Pex3p[1-50]GFP-containing membranes at another subcellular location. Similar results were obtained when GFP was replaced by β-lactamase as reporter protein. To get further insight in the donor membrane compartment of the Pex3p[1-50]-vesicles, we determined whether ER-resident proteins could be detected in, or associated with, the Pex3p[1-50]-vesicles by immunocytochemistry. Initial studies were performed using antisera raised against S. cerevisiae BiP/Kar2p or Sec63p that cross-react with the H. polymorpha orthologues. However, these antisera showed insufficient specificity and/or labelling intensity in the immunocytochemical experiments, also on normal ER. Subsequently, we made use of an artificial marker for the H. polymorpha ER lumen. A hybrid protein, consisting of the N-terminal 30 amino acids of H. polymorpha BiP (van der Heide et al., 2002), containing its ER sorting signal, and GFP was synthesised in HF290 cells (pex3::P_AMO–BiP[1-30]GFP::P_AOX–PEX3[1-50]β-lactamase) under control of the amine oxidase promoter. Fluorescence microscopic analysis of glucose/ethylamine-grown HF290 cells showed distinct staining of the nuclear envelope together with small patches at the cellular periphery (Fig. 6D). Immunocytochemical analysis showed that in these cells, α-GFP-specific labelling was confined to the nuclear membrane and the lateral ER (Fig. 6C). Next, these cells were shifted to glycerol/methanol/ammonium sulphate-medium, inducing the synthesis of Pex3p[1-
β-lactamase and fully repressing further synthesis of BiP[1-30]GFP. Four hours after the shift, these cells were prepared for immunocytochemistry. As expected, synthesis of Pex3p[1-50]β-lactamase caused the development of Pex3p[1-50]vesicles at the nuclear membrane. These vesicles contained both the Pex3p[1-50]β-lactamase hybrid protein as well as endogenous Pex14p as shown by double-labelling experiments (Fig. 6E). The Pex3p[1-50]vesicles also contain significant α-GFP-specific labelling (Fig. 6F, 5-nm gold) suggesting that the artificial marker for the nuclear membrane/ER had incorporated in these vesicles under conditions that its synthesis was fully repressed. Taken together, these data suggest that the Pex3p[1-50]vesicles may be derived from the nuclear membrane/ER.

**Fig. 6.** Pex3p[1-50]vesicles arise at the nuclear membrane. A,B, electron microscopical analysis of HF245 (pex3::PAOX::PEX3::PAMO::PEX3[1-50]GFP) cells 1 h after a shift from glucose/ammonium sulphate- to glucose/ethylamine-medium. A, Morphology; B, immunolocalisation of Pex3p[1-50]GFP using antibodies against GFP. C-F, ER-localised BiP[1-50]GFP colocalises with Pex3p[1-50]vesicles. C,D, HF290 (pex3::PAMO::BiP[1-30]GFP::PAOX::PEX3[1-50]β-lactamase) cells were grown in glucose/ethylamine-medium and analysed for the location of BiP[1-30]GFP by immunocytochemistry using α-GFP antibodies (C) and fluorescence microscopy (D, top panel, bright field image; bottom panel, fluorescence image). E,F, glucose/ethylamine grown HF290 cells (see C,D) were shifted to glycerol/methanol/ammonium sulphate-medium and after 4 h of growth analysed by immunocytochemistry for the location of Pex3p[1-50] (α-β-lactamase and 5-nm GAR-gold) and Pex14p (α-Pex14p and 15-nm GAR-gold) (E) or BiP[1-30]GFP (α-GFP and 5-nm GAR-gold) and Pex14p (α-Pex14p and 15-nm GAR-gold) (F). Abbreviations: M, mitochondrion, N, nucleus. The arrow in A indicates the Pex3[1-50]vesicle cluster. The arrows in F indicate the 5-nm gold particles specific for BiP[1-30]GFP. Bar = 0.5 µm (A-C), 5 µm (D) and 0.2 µm (E,F).
**Pex3p[1-50]**-vesicles are the target for normal peroxisome development

Since the Pex3p[1-50]-vesicles have peroxisomal characteristics, the next question we investigated is whether they can act as precursors for peroxisome biogenesis after re-introduction of Pex3p in these cells. HF245 cells were pre-cultivated in glucose/ethylamine-medium to induce Pex3p[1-50]-vesicle formation. Subsequently, these cells were incubated for 30 minutes in glucose-ammonium sulphate-medium to deplete the amine-induced mRNA's. After this incubation, the Pex3p[1-50]GFP mRNA level had dropped approximately 1,000-fold as determined by RT-PCR (data not shown). Next, the HF245 cells were transferred to methanol/ammonium sulphate-medium, thus inducing Pex3p synthesis under conditions that fully repress Pex3p[1-50]GFP synthesis. Samples were taken at regular time intervals from the HF245 culture and analysed both biochemically and microscopically. Fig. 5B shows that after 1 hour of incubation of these cells in fresh methanol/ammonium sulphate-medium, Pex3p is readily detectable at levels which are slightly higher than those in WT cells grown on glucose/ammonium sulphate (comparison to WT not shown). The high initial levels of Pex3p[1-50]GFP at t=0 (glucose/ethylamine) only gradually decreased after the shift (Fig. 5B), suggesting that under these conditions no active degradation occurs of the Pex3p[1-50]-vesicles. To analyse the mode and kinetics of peroxisome re-appearance, samples taken at various time points after the shift of cells to methanol were prepared for electron microscopical analyses. As expected, the glucose/ethylamine-grown inoculum cells (t=0) solely harboured GFP-containing vesicles and lacked peroxisomes (not shown, cf. Fig. 1). Four to six hours after the shift, some vesicles within the Pex3p[1-50]-membrane clusters had increased in size (Fig. 7A,B). Immunocytochemical staining experiments revealed that AOX protein was present in these enlarged compartments (Fig. 7B, inset). In contrast, Pex3p[1-50]GFP (Fig. 7A, inset) and Pex3p (Fig. 7B) were present throughout the whole population of vesicles, not restricted to the membranes of the enlarged vesicles. This suggests that all vesicles apparently initially accumulated Pex3p. However, not all vesicles developed into peroxisomes as indicated by the observation that after prolonged incubation of strain HF245 in methanol-media, relatively few (3-6) peroxisomes had developed (Fig 7C,D) compared to the number of vesicles that were originally present. Anti-GFP- (Fig. 7C) and Pex3p- (Fig. 7D) dependent labelling was observed on the membranes of these organelles that were characterised by the presence of AOX protein (Fig. 7D inset). The presence of the GFP marker protein on peroxisomes could also be observed by fluorescence microscopy (Fig. 7C, inset). Since the expression of Pex3p[1-50]GFP was fully
repressed under these conditions, these data suggest that the newly formed peroxisomes had developed from the Pex3p\(^{[1-50]}\)-GFP-containing vesicles. Subsequently, biochemical experiments were performed on HF245 cells, grown for 22 h after a shift to from glucose/ethylamine- to methanol/ammonium sulphate-medium. Upon gradient centrifugation of cell homogenates minor, but significant amounts of Pex3p\(^{[1-50]}\)-GFP were detected in the peroxisomal peak fraction (at 54% sucrose), where also AOX protein had accumulated (Fig. 8). Taken together,
these data suggest that the Pex3p[1-50]-vesicles can act as a template for peroxisome development after subsequent re-introduction of Pex3p.

**Complementation of pex3 cells by full length Pex3p initiates with a single peroxisome.**

In control experiments, we analysed peroxisome recovery in pex3 cells upon complementation by the PEX3 gene in the absence of Pex3p[1-50]-vesicles. Electron microscopy showed that peroxisomes arose within 1 h after the shift of cells to inducing conditions. Invariably, only a single organelle was formed, located in the vicinity of the nuclear membrane (Fig. 9). The organelle was characterised by the presence of Pex3p (Fig. 9B) and AOX (Fig. 9C). However, we never observed any proliferation of vesicles or additional membranes at the initial hours of peroxisome re-introduction comparable to Pex3p[1-50]-producing cells. This indicates that the morphological events of peroxisome re-introduction in pex3 cells significantly differ from those in similar cells that produce Pex3p[1-50] protein.
Discussion

Pex3p is a peroxisomal membrane protein essential for peroxisome biogenesis and maintenance of the organellar membrane (Höhfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 1996; Hettema et al., 2000; South et al., 2000). Previously, we reported that the first 50 amino acids of Pex3p are sufficient to target a reporter protein to the peroxisomal membrane (Baerends et al., 1996; Baerends et al., 2000). Here we show that the same fragment of the protein is capable of inducing vesicle formation in pex3 cells. These vesicles develop in the vicinity of the nuclear membrane and preferentially served as templates for peroxisome development upon subsequent re-introduction of the full-length Pex3p. These data imply that the Pex3p[1-50]-vesicles may be considered as an incomplete peroxisomal compartment that may be ‘trapped’ in its maturation because of the absence of essential functions in the missing C-terminal part (amino acids 51-457) of Pex3p.

The current view of peroxisome proliferation predicts that new peroxisomes form from pre-existing ones (Lazarow and Fujiki, 1985). However, this model can not explain some recent observations. Specifically, newly formed peroxisomes in peroxisome-deficient cells lacking any detectable peroxisomal (membrane) remnants

Fig. 9  Peroxisomes arise at the nuclear membrane upon complementation of pex3 cells. A-C electron microscopical analysis of RBG17 (pex3::P_AOX::PEX3) cells 2 h after a shift from glucose/ethylamine-medium to methanol/ammonium sulphate-medium. A. morphology; B, C, immunolocalisation of Pex3p (B) and AOX (C). Abbreviations: N, nucleus, V, vacuole. The arrow in A indicates a newly-formed peroxisome. Bar = 0.5 µm.
(human and yeast \textit{pex3} cells, human \textit{pex16}, human and \textit{S. cerevisiae pex19} cells) require an alternative membrane origin upon introduction of the complementing gene. Also, in \textit{Y. lipolytica} and in plant cells, several peroxisomal membrane proteins have been proposed to travel via the ER or an ER-like compartment before reaching the peroxisome (Titorenko and Rachubinski, 1998; Mullen et al., 1999). Whether this mechanism is generally valid, remains to be elucidated. In \textit{H. polymorpha} an ER-to-peroxisome sorting pathway may exist, exemplified by the finding that a hybrid protein consisting of the 16 N-terminal amino acids of Pex3p fused to a reporter protein (PTS1-less catalase) is targeted to the nuclear membrane (Baerends et al., 1996). The Pex3p\textsubscript{[1-50]}-GFP-induced vesicles described in this study may concentrate at certain subdomains of the apparent donor membrane compartment, the nuclear membrane. Remarkably, upon induction solely the vesicles displayed fluorescence; invariably no nuclear or ER fluorescence was detected. The finding that only vesicles showed fluorescence lends support to the notion that they may not or hardly contain typical ER characteristics. Further evidence that the Pex3p\textsubscript{[1-50]}-vesicles may indeed originate from the endomembrane system comes from the observation that they contained an overproduced ER-lumen protein, BiP\textsubscript{[1-30]}GFP.

The Pex3p\textsubscript{[1-50]}-vesicles contain at least one other peroxin, Pex14p, a protein that is missorted to mitochondria in \textit{pex3} cells (Baerends et al., 2000; South et al., 2000). Because most peroxins in \textit{H. polymorpha} are low abundant and therefore difficult to detect at endogenous levels, we have not been able to show conclusively that other peroxins are also present on these vesicles. The Pex3p\textsubscript{[1-50]}-vesicles have, however, also acquired a typical property of \textit{WT H. polymorpha} peroxisomes in that they are susceptible to selective degradation (Veenhuis et al., 1996).

Interestingly, also in mammalian \textit{pex3} cells a hybrid protein consisting of the N-terminal 40 amino acids of rat Pex3p and GFP is observed in vesicular structures (Ghaedi et al., 2000). These vesicles were however not further characterized, thus it remains to be determined whether also these structures have peroxisomal features. The key question to be answered was: are the Pex3p\textsubscript{[1-50]}-vesicles a preferred target for peroxisome re-introduction upon synthesis of Pex3p? Using an \textit{H. polymorpha} strain, in which vesicle-formation and peroxisome biogenesis can be separately regulated, we were able to show that the vesicles population accumulated Pex3p, while only few of these incorporated the peroxisomal matrix protein AOX. The possibility that the synthesis of Pex3p itself induces formation of new vesicle-clusters which mix with the pre-existing Pex3p\textsubscript{[1-50]}GFP vesicles is not very likely, because such vesicles were not observed when under identical conditions peroxisomes were
re-introduced in pex3 cells in the absence of Pex3p[1-50]-vesicles (Baerends et al., 1996) (see also Fig. 9).

Our data support the notion that 1) a Pex3p-receptor moiety is likely to exist on the endomembrane system of pex3 cells and, subsequently, on the Pex3p[1-50]-induced vesicles and 2) Pex3p[1-50]-vesicles may act as a template for the assembly of peroxisomes. Most likely, incorporation of Pex3p solely does not restore the import of peroxisomal matrix proteins into these vesicles and hence, a further development of these vesicles (e.g. by the uptake of other essential proteins) is required for this. The details of this process, however, remain to be resolved. It can be envisaged that the endogenous levels of such proteins may be too low to complement all vesicles and, in line with this, the transformation of some of the vesicles into peroxisomes may simply occur by chance.

In recent studies Titorenko and co-workers described the function of pre-peroxisomal vesicles in peroxisome biogenesis in WT Y. lipolytica cells (Titorenko et al., 2000; Titorenko and Rachubinski, 2001). Five subpopulations (P1-P5) with different biochemical characteristics were discriminated in a high-speed (200,000x g)-pellet fraction of a post-nuclear supernatant after removing the mature peroxisomes during a low-speed (20,000x g) centrifugation step. After a Pex1p- and Pex6p-dependent fusion of P1 and P2, they found that the resultant P3 was a precursor for P4, leading via P5 to mature peroxisomes, implying a prescribed maturation machinery for peroxisomes to become physiologically functional.

Other researchers have questioned the existence of vesicle-mediated, ER-to-peroxisome sorting pathways. Experiments on S. cerevisiae Pex15p, suggested that this protein was sorted via the ER, primarily because overproduction of the protein in WT cells caused massive overproliferation of ER membranes (karmellae; (Elgersma et al., 1998). In a recent study however it was shown that endogenous Pex15p remains cytosolic in pex3 cells whereas overproduced Pex15p again, gave rise to karmellea formation in these cells (Hettema et al., 2000). This led the authors to conclude that the nuclear and ER localisation of the protein, as well as the karmellea formation, may represent an artefact due to Pex15p overproduction.

In human cells, no evidence was obtained for routing of Pex16p via the ER. Like pex3 cells, human cells lacking functional Pex16p, do not contain peroxisomal ghosts. Re-appearance of peroxisomes in pex16-mutant cells upon re-introduction of the PEX16 gene was not inhibited by BFA and occurred normally at 15°C, conditions that block COPII-mediated protein exit from the ER and COPI-mediated transport from the ER/Golgi intermediate compartment, respectively (South and Gould, 1999).
Essentially similar results have recently been reported for peroxisome rescue in human *pex3* cells upon reintroduction of the complementing gene (South et al., 2000). These authors proposed a two-pathway model of peroxisome biogenesis. These include one pathway confirming to the widely accepted view that peroxisomes arise by the growth and division of pre-existing ones (Lazarow and Fujiki, 1985), and an alternative pathway by which peroxisomes form from a preperoxisomal vesicle. Their analyses, however, gave no clue as to whether such pre-peroxisomal structure exists or whether peroxisomes arise from the endomembrane system.

In WT *H. polymorpha* cells we have at present no direct evidence for a constitutive process invoking a role of ER vesicles in peroxisome biogenesis. However, our present observations suggest that specific vesicles derived from the endomembrane system or nuclear membrane may develop into normal peroxisomes. These data lend support to the view that in WT conditions sorting of Pex3p via the ER might be redundant and that, upon induction by growth compounds, peroxisomes normally develop by growth and fission. However, in cases peroxisomes are lost, e.g. due to chemical-induced damage or failure in inheritance, formation of the organelles might be rescued and initiated at the endomembrane system.

**Acknowledgements**

We thank Dr. W-H. Kunau (Ruhr University, Bochum, Germany) for the generous gift of antibodies against GFP and ADH and Dr. R. Schekman (University of California, Berkely, USA) for the generous gift of antibodies against Sec63p. This work was supported by a PULS grant from the Netherlands Organisation for Scientific Research through the Earth and Life Science Foundation to KNF. GJH and AMK were supported by the Netherlands Organisation for Scientific Research through the Earth and Life Science Foundation and the Netherlands Technology Foundation. RJSB was supported by the EC.
References


Chapter 4

