Normal Truncated Peroxisome Development from Vesicles Induced by Truncated* \textit{Hansenula polymorpha} Pex3p

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We show that the synthesis of the N-terminal 50 amino acids of Pex3p (Pex3p_{1-50}) in \textit{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 reintroduction of the full-length Pex3p protein. We discuss the significance of this finding in relation to peroxisome reinsertion, e.g. in case peroxisomes are lost due to failure in inheritance.

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. Yeast are favorable model organisms to study peroxisome biogenesis because of a number of properties. Firstly, peroxisome proliferation can be strictly dependent on the organism, the environmental conditions, and the physiological state of the cell. Yeast 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) (1, 2). 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 (per)-deficient mutants that are unable to utilize either oleate or methanol for growth (3–5). With few exceptions (see below), these \textit{pex} mutant cells still contain peroxisomal membrane remnants (“ghosts”). Through genetic complementation, 23 \textit{PEX} genes have currently been characterized (5). Data base searches using the yeast genes have presently revealed 13 human orthologues of yeast \textit{PEX} genes. Mutations in 11 of these \textit{PEN} genes have been characterized 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 (6, 7).

The analysis of the \textit{PEN} genes and the peroxins they encode has predominantly shed light on the molecular components involved in peroxisomal matrix protein import (reviewed in Refs. 4, 5). 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 synthesized on free polysomes in the cytosol and are posttranslationally transported to the peroxisome (8). Together with morphological studies on peroxisome proliferation, these data have led to the hypothesis that peroxisomes develop by fission from preexisting ones (9). 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 (10–13).

Notably, the prevailing model of budding from preexisting peroxisomes cannot explain the reassembly of peroxisomes in mutant cells lacking any peroxisomal remnants (yeast and human \textit{pex3}, \textit{H. polymorpha per13–6}‡, \textit{human pex16}, and \textit{Saccharomyces cerevisiae} and human \textit{pex19}) upon functional complementation of such cells (14–21). Several recent studies have assessed the question of the origin of the peroxisomal membrane upon reappearance of the organelles in \textit{pex}-mutants lacking peroxisomal membranes. We studied this process in a temperature-sensitive \textit{pex} mutant of the yeast \textit{H. polymorpha} (15). 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 (20), Matsuzono \textit{et al} (19), and South \textit{et al} (21) studied the reappearance of peroxisomes in human cells defective for \textit{PEX16}, \textit{PEX19}, and \textit{PEX3}, respectively. Peroxisomal remnants were absent in these cells also, but intact organelles were assembled upon reintroduction of the comple-

* This work was supported by a PULS Grant from the Netherlands Organization for Scientific Research through the Earth and Life Science Foundation (to K. N. F.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must be the molecular basis for inherited peroxisome biogenesis disorders, including Zellweger Syndrome, neonatal adrenoleukodystrophy, infantile Refsum’s disease and rhizomelic chondrodysplasia punctata (6, 7).

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1 The abbreviations used are: ER, endoplasmic reticulum; GFP, green fluorescent protein; WT, wild type; MES, 4-morpholineethanesulfonic acid; PNS, postnuclear supernatant.
menting gene. As suggested before by Waterham et al. (15), these studies revealed that peroxisomes do not necessarily derive from preexisting 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 reintroduction of full-length Pex3p, a peroxosomal 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 Pex3p1–50 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 (22) containing either 0.5% (w/v) glucose, 0.5% (v/v) methanol, or a mixture of 0.1% (v/v) glyceral and 0.5% methanol as carbon and energy source in combination with 0.25% (w/v) ammonium sulfate or 0.25% (w/v) ethylamine as sole nitrogen sources. For growth in 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.

**Peroxisome Development from Vesicles**

**RESULTS**

**Synthesis of Pex3p1–50 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 reappear in such cells upon reintroduction of the complementing _PEX3_ gene (16). In an attempt to shed light on the origin of these organelles, we introduced hybrid genes encoding N-terminal

**TABLE I**

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<th>Strain</th>
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<tr>
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<td>RBG1</td>
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</tr>
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fragments of Pex3p (containing its putative peroxisomal targeting signal) and a reporter gene (GFP or β-lactamase) in pex3 cells and determined the subcellular location of the gene products. Pex3 cells producing the Pex3p1–50GFP 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; Ref. 27). 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. Ref. 16) or in WT cells. These membrane structures were the subcellular site of the Pex3p1–50GFP hybrid protein as was shown by immunocytochemistry using α-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 Pex3p1–50GFP protein was produced by the PEX3-promoter element, though less abundantly. These data show that synthesis of the 50 N-terminal amino acids of Pex3p fused to α-GFP causes the formation of vesicles from peroxisomal remnants (ghosts) and mitochondrial cytochrome c oxidase activity (Fig. 2). Western blot analysis was used to determine the location of alcohol oxidase (AOX), Pex3p1–50GFP, mitochondrial porin, endoplasmic reticulum Sec63p, and peroxin Pex14p. All membrane-bound marker proteins co-localize in the top fractions of the gradient.

To further analyze the possible peroxisomal nature of the Pex3p1–50 vesicles, we investigated whether these structures were susceptible to selective degradation, comparable with WT peroxisomes (37) and peroxisomal remnants (ghosts) in methanol-induced H. polymorpha cells (38). To this end, HF75 (WT: Pex3p1–50GFP) and HF78 cells (pex3::Pox–Pex3p1–50GFP) were grown in glycerol/methanol-containing media to induce peroxisome and Pex3p1–50 vesicle formation, respectively. Next, these cells were transferred to fresh glucose media, thus repressing peroxisome and vesicle formation, and the fate of AOX and GFP was followed in time. As expected, in HF75 control cells both the level of peroxisomal AOX and Pex3p1–50GFP decreased significantly (Fig. 3C), and after

![Fig. 1. Synthesis of Pex3p1–50GFP causes vesicle formation in pex3 cells.](image)

![Fig. 2. Pex3p1–50 vesicles migrate to low-density fractions after sucrose density gradient centrifugation.](image)
Fig. 3. Pex3p1–50 vesicles harbor peroxisomal characteristics. A, a sucrose density gradient with an adapted sucrose density profile was used to fractionate an organellar pellet fraction prepared from glycerol/methanol-grown HF78 cells. Individual fractions were analyzed for sucrose density (+), protein concentration (○), and mitochondrial cytochrome c oxidase activity (▲). Western blot analysis was used to determine the location of Pex3p1–50 GFP (top panel). Peak fractions of mitochondria were found in fraction 8, whereas Pex3p1–50 GFP was most abundant in fraction 14. B, Western blot analysis of fractions 8, 12, and 14 for Pex3p1–50 GFP, Pex14p, porin, and Sec63p. Pex14p cofractionates with the Pex3p1–50 GFP vesicles. C, selective degradation of Pex31–50 vesicles; strains HF75 (WT::PAMO–PEX31–50GFP) and HF78 (pex3::PAMO–PEX31–50GFP) were grown in glycerol/methanol medium to the late-logarithmic phase of growth and shifted to glucose medium, and at selected time points samples were taken. Whole cell extracts, representing equal culture volumes, were analyzed for the levels of AO and Pex3p1–50 GFP by Western blotting.

Pex3p1–50 Vesicles Develop at the Nuclear Membrane—Next, we investigated the origin of the vesicles induced upon production of Pex3p1–50 GFP. To this end, we constructed a strain, HF245, in which Pex3p1–50 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 (PAMO) together with the gene encoding Pex3p1–50 GFP under control of the amine-inducible amine oxidase promoter (PAMO) (Fig. 5A). HF245 cells were shifted from PAMO-repressing conditions (glucose/ammonium sulfate) to media that induce the PAMO (glucose/ethanolamine). 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, Pex3p1–50 GFP was solely detectable at these membranes (Fig. 6B). We never observed the development of a cluster of Pex3p1–50 GFP-containing membranes at another subcellular location. Similar results were obtained when GFP was replaced by β-lactamase as reporter protein. To further get insight in the donor membrane compartment of the Pex3p1–50 vesicles, we determined whether ER-resident proteins could be detected in, or associated with, the Pex3p1–50 vesicles by immunocytochemistry. Initial studies were performed using antiseras raised 4 h of induction in glucose medium, a 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 (39). In contrast, the level of Pex3p1–50 GFP decreased with similar kinetics as observed for HF75 cells, suggesting that the Pex3p1–50 GFP vesicles were actively degraded after a shift of HF78 cells to glucose medium. Taken together, these data suggest that the Pex3p1–50 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 Pex31–50 vesicles. Immunolabeling of the organellar fraction loaded onto the sucrose gradient analyzed in Fig. 3A using antibodies against GFP (A) and Pex14p (B) is shown. Typical clusters of small vesicles were specifically labeled using these antibodies. M, mitochondrion. Bar = 0.2 μm.
against *S. cerevisiae* BiP/Kar2p or Sec63p that cross-react with the *H. polymorpha* orthologues. However, these antisera showed insufficient specificity and/or labeling intensity in the immunocytochemical experiments and 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 and containing its ER sorting signal, and GFP was synthesized in HF290 cells (*pex*3::*PAOX::PEx31-GFP::*PEX31-GFP) cells 1 h after a shift from glucose/ammonium sulfate to glycerol/methanol/ammonium sulfate medium. A and B, 6 h after the shift; C and D, 16 h after the shift. A, morphology; B-D and insets, immunolocalization of Pex3p1-GFP (A inset, C); B and D, Pex3p; and B inset and D inset, AOX. Pex3p and AOX are sorted to the Pex3p1-GFP vesicle clusters (B). 16 h after the shift, significant amounts of Pex3p1-GFP is found on the peroxisomal membrane (C). The inset in C shows a fluorescent image of HF245 cells 14 h after the shift to methanol/ammonium sulfate medium. *M*, mitochondrion; *N*, nucleus; *P*, peroxisome; *V*, vacuole. The arrow in A and E indicates the Pex3p1-50 vesicle cluster. Bar = 0.5 μm (similar magnifications were used for A-D).

**Fig. 6.** Pex3p1-50 vesicles arise at the nuclear membrane. *A* and *B*, electron microscopical analysis of HF245 (*pex*3::*PAOX::PEx31-GFP) cells 1 h after a shift from glucose/ammonium sulfate to glucose/ethylamine medium. *A*, morphology. *B*, immunolocalization of Pex3p1-GFP using antibodies against GFP. *C-F*, ER-localized BiP1-GFP co-localizes with Pex31-50 vesicles. *C* and *D*, HF290 (*pex*3::*PAOX::BiP1-30 GFP::*PEX31-GFP) cells were grown in glucose/ethylamine medium and analyzed for the location of BiP1-GFP by immunocytochemistry using α-GFP antibodies (C) and fluorescence microscopy (D, top panel, bright field image; bottom panel, fluorescence image). *E* and *F*, glucose/ethylamine-grown HF290 cells (see *C* and *D*) were shifted to glycerol/methanol/ammonium sulfate medium and after 4 h of growth analyzed by immunocytochemistry for the location of Pex3p1-GFP, using α-GFP antibodies (E) and fluorescence microscopy (F). 

**Fig. 7.** Pex3p1-50 vesicles are the template for peroxisome biogenesis. Electron microscopical analysis of HF245 (*pex*3::*PAOX::PEx31-GFP) cells shifted from glucose/ethylamine medium to glycerol/methanol/ammonium sulfate medium. *A* and *B*, 6 h after the shift; *C* and *D*, 16 h after the shift. *A*, morphology; *B-D* and insets, immunolocalization of Pex3p1-GFP (*A* inset, *C*); *B* and *D*, Pex3p; and *B* inset and *D* inset, AOX. Pex3p and AOX are sorted to the Pex3p1-GFP vesicle clusters (*B*). 16 h after the shift, significant amounts of Pex3p1-GFP is found on the peroxisomal membrane (*C*). The inset in *C* shows a fluorescent image of HF245 cells 14 h after the shift to methanol/ammonium sulfate medium. *M*, mitochondrion; *N*, nucleus; *P*, peroxisome; *V*, vacuole. The arrow in *A* and *E* indicates the Pex3p1-50 vesicle cluster. Bar = 0.5 μm (similar magnifications were used for *A*-D).

*Pex3p1-50 Vesicles Are the Target for Normal Peroxisome Development*—Since the Pex3p1-50 vesicles have peroxisomal characteristics, the next question we investigated is whether...
they can act as precursors for peroxisome biogenesis after reintroduction 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 min in glucose-ammonium sulfate medium to deplete the amine-induced mRNAs. After this incubation, the Pex3p_{1–50} GFP mRNA level had dropped ~1,000-fold as determined by reverse transcription-PCR (data not shown). Next, the HF245 cells were transferred to methanol/ammonium sulfate 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 analyzed both biochemically and microscopically. Fig. 5B shows that after 1 h of incubation of these cells in fresh methanol/ammonium sulfate medium, Pex3p is readily detectable at levels that are slightly higher than those in WT cells grown on glucose/ammonium sulfate medium (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 analyze the mode and kinetics of peroxisome reappearance, samples taken at various time points after the shift of cells to methanol were prepared for electron microscopic analyses. As expected, the glucose/ethylamine-grown inoculum cells (t = 0) solely harbored GFP-containing vesicles and lacked peroxisomes (not shown, cf. Fig. 1). Four to six h after the shift, some vesicles within the Pex3p_{1–50}-membrane clusters had increased in size (Fig. 7, A and B). Immunocytochemical staining experiments revealed that AO 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. 7, C and D) compared with the number of vesicles that were originally present. Anti-GFP- (Fig. 7C) and Pex3p- (Fig. 7D) dependent labeling was observed on the membranes of these organelles that were characterized by the presence of AO 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 from glucose/ethylamine medium to methanol/ammonium sulfate 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 AO 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 reintroduction of Pex3p.

Complementation of pex3 Cells by Full-length Pex3p Initiates with a Single Peroxisome—In control experiments, we analyzed 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 characterized by the presence of Pex3p (Fig. 9B) and AO (Fig. 9C). However, we never observed any proliferation of vesicles or additional membranes at the initial hours of peroxisome reintroduction comparable with Pex3p_{1–50}-producing cells. This indicates that the morphological events of peroxisome reintroduction 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 (14, 16, 17, 21, 40). Previously, we reported that the first 50 amino acids of Pex3p are sufficient to target a reporter protein to the peroxisomal membrane (16, 27). 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 reintroduction of the full-length Pex3p. These data imply that the Pex3p₁₋₅₀ 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 preexisting ones (9). However, this model can not explain some recent observations. Specifically, newly formed peroxisomes in peroxisome-deficient cells lacking any detectable peroxisomal (membrane) remnants (human and yeast pex3 cells, human pex16, human and S. cerevisiae pex19 cells) require an alternative membrane origin upon introduction of the complementing gene. Also, in 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 (10, 12). Whether this mechanism is generally valid, remains to be elucidated. In 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 (16). The Pex3p₁₋₅₀-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 Pex3p₁₋₅₀ vesicles may indeed originate from the endomembrane system comes from the observation that they contained an overproduced ER-lumen protein, BiP₁₋₅₀-GFP.

The Pex3p₁₋₅₀ vesicles contain at least one other peroxin, Pex14p, a protein that is missorted to mitochondria in pex3 cells (21, 27). Because most peroxins in 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 Pex3p₁₋₅₀ vesicles have, however, also acquired a typical property of WT H. polymorpha peroxisomes in that they are susceptible to selective degradation (38).

Interestingly, also in mammalian pex3 cells a hybrid protein consisting of the N-terminal 40 amino acids of rat Pex3p and GFP is observed in vesicular structures (41). 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 Pex3p₁₋₅₀ vesicles a preferred target for peroxisome reintroduction upon synthesis of Pex3p? Using a 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 AO. The possibility that the synthesis of Pex3p itself induces formation of new vesicle-clusters that mix with the preexisting Pex3p₁₋₅₀-GFP vesicles is not very likely, because such vesicles were not observed when under identical conditions peroxisomes were reintroduced in pex3 cells in the absence of Pex3p₁₋₅₀ vesicles (16) (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 Pex3p₁₋₅₀-induced vesicles and 2) Pex3p₁₋₅₀ 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 preperoxisomal vesicles in peroxisome biogenesis in WT Y. lipolytica cells (13, 42). Five subpopulations (P1–P5) with different biochemical characteristics were discriminated in a high-speed (200,000 × g) pellet fraction of a postnuclear supernatant after removing the mature peroxisomes during a low-speed (20,000 × 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 Pex16p, 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; Ref. 43). In a recent study, however, it was shown that endogenous Pex15p remains cytosolic in pex3 cells, whereas overproduced Pex15p again, gave rise to karmella formation in these cells (40). This led the authors to conclude that the nuclear and ER localization of the protein, as well as the karmellae formation, may represent an artifact 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. Reappearance of peroxisomes in pex16-mutant cells upon reintroduction of the PEX16 gene was not inhibited by brefeldin A and occurred normally at 15 °C, conditions that block COPII-mediated protein exit from the ER and COPII-mediated transport from the ER/Golgi intermediate compartment, respectively (20). Essentially similar results have recently been reported for peroxisome rescue in human pex3 cells upon reintroduction of the complementing gene (21). These authors proposed a two-pathway model of peroxisome biogenesis. These include one pathway confirming the widely accepted view that peroxisomes arise by the growth and division of preexisting ones (9) and an alternative pathway by which peroxisomes form from a preperoxisomal vesicle. Their analyses, however, gave no clue as to whether such preperoxisomal 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 involving 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 where 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.

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