p24 proteins play a role in peroxisome proliferation in yeast

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A B S T R A C T
Emp24 is a member of the p24 protein family, which was initially localized to the endoplasmic reticulum, Golgi and COP vesicles, but has recently shown to be associated with Saccharomyces cerevisiae peroxisomes as well. Using cell fractionation and electron- and fluorescence microscopy, we show that in the yeast Hansenula polymorpha, Emp24 also associates with peroxisomes. In addition, we show that peroxisome numbers are strongly decreased in H. polymorpha cells lacking two proteins of the p24 complex, Emp24 and Erp3. Detailed fluorescence microscopy analyses suggest that emp24.erp3 cells are disturbed in peroxisome fission and inheritance.

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1. Introduction

A detailed quantitative mass spectrometry study of highly purified peroxisomal fractions obtained from Saccharomyces cerevisiae revealed that several presumed non-peroxisomal proteins showed in fact a dual localization and were associated with peroxisomes as well [1]. The most prominent ones that were identified in this proteomics study were Rho1, Gpd1 and Emp24. Rho1 was initially localized to the plasma- and endomembranes, where it plays a role in regulating polarized growth [2]. Interestingly, this protein also appeared to interact with the peroxisomal membrane proteins Pex25 and Pex30 and most likely regulates the assembly state of actin at the peroxisomal surface [1]. Gpd1 (glycerol-3-phosphate dehydrogenase) is a cytosolic enzyme that functions in a cytosolic/mitochondrial glycerol phosphate shuttle. Why a portion of this protein is associated with peroxisomes is still unknown.

Emp24, a member of the p24 protein family, has been localized to the endoplasmic reticulum (ER), Golgi-apparatus and COP vesicles [3]. Members of the p24 protein family have been implicated in COP vesicle biogenesis and function, cargo selection and regulation of vesicles transport through the secretory pathway [3]. Based on phylogenetic analysis, the p24 protein family consists of four subfamilies (α, β, γ and δ). Studies in yeast suggested that p24 complexes form heterotetramers that contain one protein of each subfamily [4]. However, studies in mammals suggested that p24 proteins also can form different kinds of dimers [5].

Previously, we reported that the genome of the yeast Hansenula polymorpha encodes four p24 proteins, one of each subfamily [6]. In a strain in which two of these genes were deleted (EMP24 and ERP3), peroxisome formation was affected. Our present study builds on to this observation and addresses the function of these p24 proteins in peroxisome biology in more detail.

2. Materials and methods

2.1. Organisms and growth

H. polymorpha cells were grown as described previously [6]. The strains used in this study are listed in Supplementary Table 1. Strains emp24.erp3.pex3 and emp24.erp3.DsRed-SKL.GFP-SKL were obtained by crossing of respectively emp24.erp3 with pex3 and emp24.erp3 with WT.DsRed-SKL.GFP-SKL. Upon sporulation the correct strains were selected.

2.2. Construction of plasmids

The plasmids used in this study are listed in Supplementary Table 2. To construct plasmid pHIPX5.EMP24-green fluorescent protein (GFP), a DNA fragment containing the EMP24 gene was amplified using primers emp24-fusion forward (5’-CGTACGCTC-AGTAGATCTCCATGATCGATGACCTGTCATTTTCACCTGTCGCC-3’).
and emp24-fusion reverse (5’-CGACCAGATCTACGGCTTGC-CTCGAAGAATCTCTTGAG-3’). The 700 bps-PCR fragment was digested with BglII and Xhol and cloned into pANL31. The resulting plasmid was digested with BamHI and SmaI and the 1.4-kb fragment was cloned into pHIPX5, digested with the same enzymes. The resulting plasmid pHIPX5.EMP24-GFP was linearized with BsiWI and integrated into the genome of wild type (WT) resulting in strain EMK1.

Plasmid pHIPZ7.DsRed C-terminal tripeptide serine–lysine–leucine (–SKL) was constructed as follows: the NatI–BamHI fragment of pHIPZ4.DsRed-SKL containing the Hp alcohol oxidase (AOX) promoter, was exchanged with the corresponding fragment from pHIPX7, containing the Hp TEF1 promoter. The resulting plasmid was linearized with MunI and transformed into EMK1 strain to create strain EMK E8.

For the construction of plasmid pHIPZ5.PEX3-GFP, the 2.2 kb BamHI–SmaI fragment from pHOR46 containing PEX3-GFP was inserted between the BamHI–Pael (blunted) sites of pHIPZ5. For stable integration of the expression cassette into the genome of emp24.erp3.pex3 and RBG1, the plasmid was linearized using BsiWI, resulting in EMK RE16 and EMK R19, respectively.

2.3. Molecular techniques

All DNA manipulations were carried out according to standard methods. Transformation of *H. polymorpha* cells and site specific integration were performed as described [7,8]. Correct integrations were confirmed by Southern blot analysis.

2.4. Microscopy methods

For fluorescence microscopy, cells were fixed in 4% formaldehyde in 10 mM potassium phosphate buffer, pH 7.5, for 2 h on ice. Quantification experiments were performed using two independent cultures (150 cells per culture). Fluorescence images were made using a Zeiss Axiovert 135 fluorescence microscope (Zeiss Netherlands b.v., Weesp, The Netherlands) [9]. Electron microscopy and cell fractionation was performed as described before [9].

3. Results

3.1. In *H. polymorpha* cells that lack Emp24 and Erp3 peroxisome abundance is reduced

Detailed quantitative analysis of peroxisome numbers in identically grown WT cells and cells of a strain in which the *EMP24* and *ERP3* genes were deleted (designated *emp24.erp3*) (Fig. 1A and B) revealed that the average number of peroxisomes in *emp24.erp3* cells was almost twofold reduced relative to WT controls. In *emp24.erp3* cells the average number of peroxisomes per cell was 1.2 ± 0.05 relative to 2.2 ± 0.05 in WT (± represents standard error of mean). A z-test revealed that these average numbers are significantly different (*P* < 0.001). Western blot analysis showed that the reduction in peroxisome numbers was not due to strongly decreased levels of Pex3 or Pex11 (Fig. 1C), which was previously reported to result in decreased peroxisome numbers [10–12]. Also, both peroxins were normally localized to peroxisomes of *emp24.erp3* cells (data not shown).

3.2. A portion of Emp24 colocalizes with peroxisomes

To determine the subcellular localization of Emp24, a strain was constructed that produced Emp24 containing GFP at the C-terminus (Emp24-GFP) in addition to DsRed-SKL to mark peroxisomes. Subfractionation experiments revealed that the bulk of the Emp24-GFP co-localized with the ER marker protein Sec63 (Fig. 2A, fractions 13–15). However, a portion of the protein was present at high density (fractions 5 and 6), where peroxisomes sediment. A peroxisomal localization was confirmed by fluorescence microscopy analysis, which revealed that Emp24-GFP was predominantly localized to the ER/nuclear envelope, but a portion co-localized with peroxisomes often in a spot at the organelle (Fig. 2B). A similar result was obtained by electron microscopy (immunolabelling using antibodies against GFP; Fig. 2C and D), which indicated that gold particles were predominantly present at the nuclear envelope (Fig. 2D), but also at the peroxisomal membrane (Fig. 2C and D).
3.3. Emp24 and Erp3 are not required for re-introduction of peroxisomes in pex3 cells

In *H. polymorpha* pex3 cells peroxisomal structures and membranes are completely absent. However, upon reintroducing the PEX3 gene new organelles are formed from the ER [13]. To analyze whether Emp24 and Erp3 are required for this process, we constructed a strain in which *EMP24*, *ERP3*, and *PEX3* were deleted (designated *emp24.erp3.pex3*). In this strain the *PEX3-GFP* gene was introduced under control of the inducible amine oxidase promoter (P<sub>AMO</sub>). *Emp24.erp3.pex3.P<sub>AMO</sub>PEX3-GFP* cells did not contain GFP fluorescence, when the cells were grown at conditions that fully repress *P<sub>AMO</sub>* (Fig. 3B, t = 0 h). After shifting these cells to media that induce *P<sub>AMO</sub>* and *P<sub>AMO</sub>PEX3-GFP* gene was introduced under control of the inducible amine oxidase promoter (P<sub>AMO</sub>). As expected *emp24.erp3.pex3.P<sub>AMO</sub>PEX3-GFP* cells did not contain GFP fluorescence, when the cells were grown at conditions that fully repress *P<sub>AMO</sub>* (Fig. 3B, t = 0 h). After shifting these cells to media that induce *P<sub>AMO</sub>* the first bright fluorescent spot was observed within 1 h of cultivation (Fig. 3B, t = 1 h), similar as observed in *pex3* control cells in which *P<sub>AMO</sub>PEX3-GFP* was induced (Fig. 3A [13]). During prolonged cultivation these spots increased in size and subsequently in number in *pex3.P<sub>AMO</sub>PEX3* controls (Fig. 3A), but not in *emp24.erp3.pex3.P<sub>AMO</sub>PEX3-GFP* cells (Fig. 3B), which generally contained a single organelle similar as in *emp24.erp3* cells. Based on these observations we conclude that Emp24 and Erp3 are not essential for the re-introduction of peroxisomes in *pex3* cells.

3.4. Deletion of EMP24 and ER3 leads to defects in peroxisome inheritance and fission

In budding methanol-grown *H. polymorpha* WT cells at least one peroxisome is inherited to newly developing buds, whereas the remaining organelles are retained in the mother cells [6]. Fluorescence microscopy analysis of *emp24.erp3* cells suggested that peroxisome trafficking to developing buds was affected. To investigate this in more detail, the presence of peroxisomes in buds of methanol-grown *emp24.erp3* cells was quantified. As shown in Fig. 4A the number of buds that contained a peroxisome was strongly reduced in *emp24.erp3* cells relative to WT controls (30% versus 50%; these values are significantly different based on a t-test, *P* < 0.05). Organelle positioning in the budding cells was not affected as the peroxisomes normally migrated to the neck region between mother cell and bud (Fig. 4A), like in WT control cells. Also life cell
imaging did not reveal any migration of peroxisomes into the bud, nor was peroxisome fission observed (data not shown; compare Fig. 4C). These observations suggest that the buds do not inherit a peroxisome from the mother cell, but instead form new peroxisomes.

The putative new formation of peroxisomes in buds of emp24.erp3 cells was analyzed in detail using emp24.erp3.pex3 cells that produce WT levels of Pex3-GFP (Fig. 4B). In the buds that lacked a peroxisome, Pex3p-GFP first accumulated into structures, most likely ER/nuclear envelope and subsequently condensed into a clear spot (Fig. 4C). The new formation of the organelle is furthermore evident from the kymogram (Fig. 4D).

3.5. Peroxisome fission is blocked in emp24.erp3 cells

We recently showed that in H. polymorpha WT cells peroxisomes predominantly proliferate by fission, using a pulse chase approach with two different peroxisomal marker proteins [14]. To further address the apparent impairment of peroxisome fission in emp24.erp3 cells we used the same experimental approach. To this purpose, we constructed an emp24.erp3 strain, which produced two fluorescent marker proteins, GFP-SKL and DsRed-SKL, that were under control of two different and independently regulatable promoters, namely the methylamine-inducible PAMO and the methanol-inducible Paxl. Cells were pre-grown on glucose–methylamine medium, which resulted in emp24.erp3 cells that generally contained a single, small GFP-SKL marked peroxisome. Subsequently, the cells were incubated for 30 min in the presence of ammonium sulphate and in the absence of a carbon source, to repress Paxl and deplete for GFP-SKL mRNA's [14]. After this treatment, the cells were shifted to methanol–ammonium sulphate-containing media to induce Paxl–driven DsRed-SKL synthesis and peroxisome proliferation [14]. A few hours after the shift, the first DsRed signal was evident and solely accumulated in the pre-existing GFP containing peroxisome (Fig. 5A). Occasionally, a second organelle developed in the cells, but this organelle invariably only contained red fluorescence, suggesting that they did not originate by fission of the pre-existing organelle (Fig. 5B). Also, small peroxisomes present in developing buds invariably lacked green fluorescence (Fig. 5C). These observations suggest that peroxisomes in young buds of emp24.erp3 cells do not arise by fission of the pre-existing organelle present in the mother cell, as is the common mode in WT [14,15].

In H. polymorpha peroxisome fission depends on the dynamin related protein Dnm1 [14]. Moreover, overproduction of Dnm1 in

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**Fig. 3.** Emp24 and Erp3 are not required for re-introduction of peroxisomes in pex3 cells. Fluorescence microscopy showing that in pex3 control cells (A) and in emp24.erp3.pex3 cells (B) peroxisomes are formed after induction of PEX3-GFP (t = 0 h). The kinetics of organelle re-appearance is similar in both strains. Upper panels: bright field; lower panels: GFP fluorescence.

**Fig. 4.** emp24.erp3 cells are defective in peroxisome inheritance. (A) Quantification of fluorescent spots in budding emp24.erp3 and WT cells producing DsRed-SKL. Cells with buds of approx. 30% of the size of mother cells were counted. The bar represents the standard error of mean. (B) Western blot showing that the Pex3-GFP producing strain produces WT Pex3 levels. Cells of WT and the emp24.erp3.Pex3-GFP strain were grown at identical conditions. The blots were decorated with anti-Pex3p antibodies. Equal amounts of protein were loaded per lane. (C) Stills of a video (see Supplementary video) showing a budding emp24.erp3 cell producing Pex3-GFP. The newly formed bud initially lacks a peroxisome (85 min). At later time points a new peroxisome develops (115 min). Upper panel: Pex3-GFP; lower panel: overlay picture of bright field and GFP fluorescence. Numbers indicate time in minutes. (D) Kymogram [14] of different stages of the video shown in (C). The kymogram illustrates that a new peroxisomes is formed in the bud. At the left side the fluorescence of the large peroxisome in the mother cells is seen, at the right the appearance of a new organelle in the bud. The top of the kymogram represents an early time point of the video, the bottom a late time point.
EMP24 and Erv25, which belong to the p24 subfamily, whereas Erp3 belongs to the p24 γ subfamily. A recent detailed analysis of the phylogeny of the vertebrate p24 protein family revealed that p24 β and p24 γ are evolutionarily related [20]. Hence it is possible that only these two proteins, but not Erp5 and Erv25, which belong to the p24 α and δ subfamilies, respectively, play a role in peroxisome formation.

We recently showed that blocking peroxisome fission in *H. polymorpha* by deletion of DNM1 results in the presence of a single enlarged peroxisome, which forms a long extension that protrudes into the developing bud. Such extensions are not formed in *pex11* cells, which is in line with the assumption that Pex11 plays a role in peroxisome elongation [21]. As also in *emp24.erp3* cells we never observed peroxisome extensions, we suggest that in these cells also an early stage in peroxisome fission is affected.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.febslet.2009.08.040.

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