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Peroxisomal remnants in peroxisome-deficient mutants of the yeast *Hansenula polymorha*

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Abstract We have analyzed the presence of peroxisomal remnants ('ghosts') in three peroxisome-deficient (*per*) mutants of the yeast *Hansenula polymorpha*, namely Δ*per4*, Δ*per5* and Δ*per10*. Under peroxisome-inducing growth conditions peroxisomal membrane proteins (PMPs) were normally synthesized in cells of these mutants. In addition, these cells contained clusters of small membranous vesicles, which were absent in cells grown under peroxisome-repressing growth conditions. These structures displayed typical peroxisomal properties in that they proliferated upon overproduction of Per8p, the *H. polymorpha* peroxisome proliferation factor. Moreover, in Δ*per4* and Δ*per5* these vesicles were susceptible to glucose-induced proteolytic degradation.

Key words: Yeast; Peroxisome; Peroxisome-deficient mutant; Peroxisomal membrane protein

1. Introduction

Peroxisomes are essential subcellular organelles and carry out multiple functions which depend on cell type, organism and developmental stage of the organism in which they occur [1,2]. In yeast they are mainly involved in the primary metabolism of specific carbon-and/or nitrogen source used for growth. In the course of our studies on the molecular mechanisms of peroxisome biogenesis in the methylotrophic yeast *Hansenula polymorpha*, we have isolated and characterized various peroxisome-deficient mutants (*per* mutants [3]). These mutants have been identified within a collection of mutants which were defective in growth on methanol as sole carbon and energy source (Mut$^-$ phenotype). At present 28 different complementation groups have been identified, which include both constitutive and conditional (Ts) mutants; 10 *PER* genes (*PER1-PER10*) have now been cloned and characterized [4].

Analysis of the phenotype of various *H. polymorpha PER* disruption strains (Δ*per* strains) showed that such cells had a number of characteristics in common:

- Δ*per* cells are invariably Mut$^-$, but grow well on all other substrates which are known to require peroxisomal functions in wild-type cells (e.g. growth on organic nitrogen sources like D-amino acids and primary amines).
- In Δ*per* cells peroxisomal matrix enzymes are normally synthesized and active in the cytosol. In the case of high alcohol oxidase (AO) levels cytoplasmic AO crystalloids are formed, in which other matrix enzymes, except catalase, are included.
- In Δ*per* cells the peroxisomal membrane proteins (PMPs) yet studied are also normally synthesized.

This raises the question of where these PMPs are localized. Are they all in one and the same compartment ('ghosts') as they are observed in mammalian cells [5] and bakers yeast *peb2* and *peb4* cells [6]? For this reason, we have studied the presence of peroxisomal membrane remnants in detail in different *H. polymorpha per* disruption strains. The results of these studies are presented in this paper.

2. Materials and methods

2.1. Micro-organisms and growth conditions

H. polymorpha NCYC495 *leu1.1 ura3* was used to create disruption mutants of *PER4*, *PER5*, *PER9* and *PER10* by insertion of the *URA3* gene of *H. polymorpha* into these genes. This strategy resulted in *per4::URA3*, *per5::URA3*, *per9::URA3* and *per10::URA3* strains which are Leu$^-$ and deficient to grow on methanol (Mut$^-$) ([7,8]; Hilbrands et al., unpublished results). The disruption mutants were transformed with pET4, containing the *H. polymorpha PER8* gene under control of the alcohol oxidase promoter [9]. Cells were grown at 37°C in carbon-limited continuous cultures at a dilution rate of 0.1 h^{-1}, supplemented with 0.25% (w/v) glucose and 0.2% (w/v) choline [10] or 0.25% (w/v) glucose, 0.2% ammonium sulphate and 0.1% (v/v) methanol.

In order to test whether degradation of peroxisomal remnants occurs after a shift of cells to glucose-excess conditions, cells were taken from a chemostat culture and diluted into fresh batch cultures, containing 0.5% (w/v) glucose, at a density of 0.5 (measured as absorbance at 660 nm) and cultivated at 37°C. Samples were taken at 0, 0.5, 1, 2 and 4 h.

2.2. Biochemical methods

The preparation of crude extracts [10], determination of protein concentrations [11] and Western blotting [12] were performed as detailed previously. Cell fractionation was performed as described before [13], except that 1 mM PMSF and 2.5 μg ml^{-1} leupeptin were added to all solutions. The pellets obtained after differential centrifugation (30 000 × *g* pellet (P3) and 100 000 × *g* pellet (P4)) were used for purification of peroxisomal membranes by flotation in sucrose gradients [14].

2.3. Electron microscopy

Whole cells were fixed and embedded in Epon 812 or Unicryl [15]. Ultrathin Unicryl sections were labeled using polyclonal anti-Per8p antibodies raised in rabbit and goat-anti-rabbit antibodies conjugated to gold according to the instructions of the manufacturer (Amersham, UK).

3. Results

Waterham et al. [15] were the first to identify minor amounts of membranous structures in fully derepressed *H. polymorpha* Δ*per1* cells and suggested that they may represent peroxisomal remnants. We have now found comparable struc-

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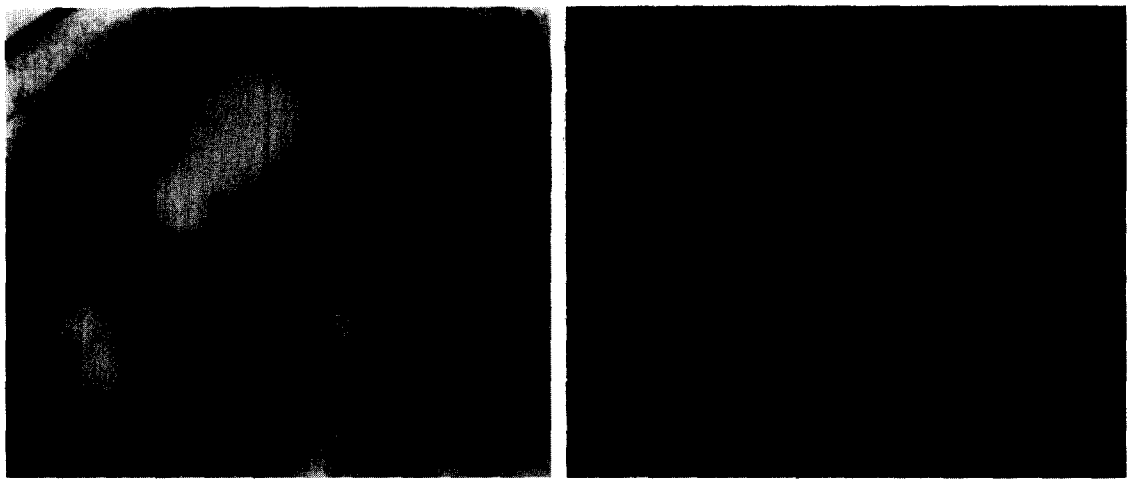

Fig. 1. (A) Characteristic membrane vesicles present in cells of Δ*per4*, incubated in methanol-containing media, labeled with anti-Per8p antibodies. After freeze-etching such membranes show largely smooth fracture faces, typical of peroxisomal membranes (B: Δ*per5*, methanol). (Inset, A) Purified vesicles from Δ*per5* overproducing Per8p. *, alcohol oxidase crystalloid; N, nucleus. The bar represents 0.5 μm.

tures in Δ*per4*, Δ*per5* and Δ*per10* (Fig. 1). As in Δ*per1* cells [15], they were invariably present in small clusters in derepressed cells, but undetectable in cells grown under peroxisome-repressing growth conditions. In freeze-etch replicas (Fig. 1B) these membranes displayed the typical smooth fracture face of intact *H. polymorpha* peroxisomes, indicating that these vesicles may indeed represent peroxisomal membrane remnants.

Attempts to purify the vesicles by conventional differential and sucrose-density centrifugation of crude cell homogenates failed due to the fact that the amounts present are too low (generally 2–5% of peroxisomal proteins are recovered this way). For this reason we investigated whether the putative peroxisomal membrane remnants could be tagged by overproduction of the homologous Per8p, an integral peroxisomal membrane protein of *H. polymorpha*, which is involved in peroxisome proliferation [8]. Western blots, prepared from crude extracts from fully derepressed cells of Δ*per4*, Δ*per5* and Δ*per10*, transformed with an expression plasmid carrying the *PER8* gene under control of the strong alcohol oxidase promoter(P_{AOX}), showed that high levels of Per8p were present in these cells, compared to untransformed WT controls (Fig. 2).

Electron-microscopical analysis of these cells showed that (i) the number of vesicles had drastically increased in all three *PER8* overexpressing mutants and (ii) that Per8p had specifically accumulated in these vesicles, as judged from immunocytochemical experiments using specific antibodies against Per8p (Fig. 3).

These results suggested that the Per8p topogenic signals are indeed recognized by these vesicles and therefore must be considered to originate from peroxisomes. Furthermore, these vesicles apparently still displayed peroxisomal functions because they proliferated similarly to normal peroxisomes during overproduction of Per8p. Hence, we studied whether these vesicles also displayed another typical peroxisomal property, namely the susceptibility to proteolytic degradation (carbon catabolite inactivation). For this purpose, fully derepressed Per8p-overproducing cells of Δ*per4*, Δ*per5* and Δ*per10* were shifted to fresh batch media supplemented with 0.5% (w/v) glucose. Western blot analysis of samples, taken at various time intervals after the shift, revealed that in Δ*per4* and Δ*per5*, but not in Δ*per10*, the amount of Per8p was rapidly reduced (Fig. 4). However, in Δ*per10* the Per8p levels remained approximately constant during the initial period after the addition of glucose. These results were confirmed by electron microscopy; in Δ*per4* and Δ*per5* cells, incubated for 30 min in fresh glucose medium, Per8p-containing vesicles were no longer detectable by immunocytochemistry. In contrast, they were readily recovered in cells of Δ*per10*, 4 h after the shift of cells to glucose (Fig. 3).

Also in cells of Δ*per9*, which has been shown not to contain peroxisomal membrane vesicles [7] and therefore studied for comparison, degradation of Per8p was not observed after the shift of derepressed cells to glucose (Fig. 4).

We have isolated the vesicles, accumulating in Per8p-overproducing cells of Δ*per4*, Δ*per5* and Δ*per10*, from homogenates of glucose/choline-grown cells using Per8p as a marker protein. After differential centrifugation the major portion of Per8p was sedimentable and accumulated in the $30\,000 \times g$ pellet (Fig. 5, lane 1). The remaining portion present in the $30\,000 \times g$ supernatant was subsequently pelleted at $100\,000 \times g$ (Fig. 5. lane 2) indicating that Per8p was sedimentable in the three *per* disruption strains. The Per8p containing peroxisomal remnants enriched in the P3 fraction were further purified by floatation centrifugation in sucrose gradients [14]. These studies revealed that Per8p migrated to lower densities, indicating that Per8p is incorporated into membranes. The peroxisomal matrix proteins catalase and amine oxidase were not detected in these fractions whereas alcohol oxidase was present at low levels (data not shown). PMPs other than Per8p could not be unequivocally demonstrated, most probably because their levels are too low to be detected by the applied methods.

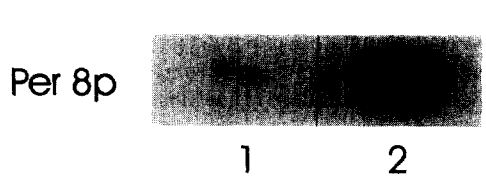

Fig. 2. Western blot, prepared from crude extracts of wild-type *H. polymorpha* (lane 1) and Δ*per5*·P_{AOX}*PER8* cells (lane 2), grown/incubated on methanol-containing media, showing the overproduction of Per8p in the constructed mutant cells.

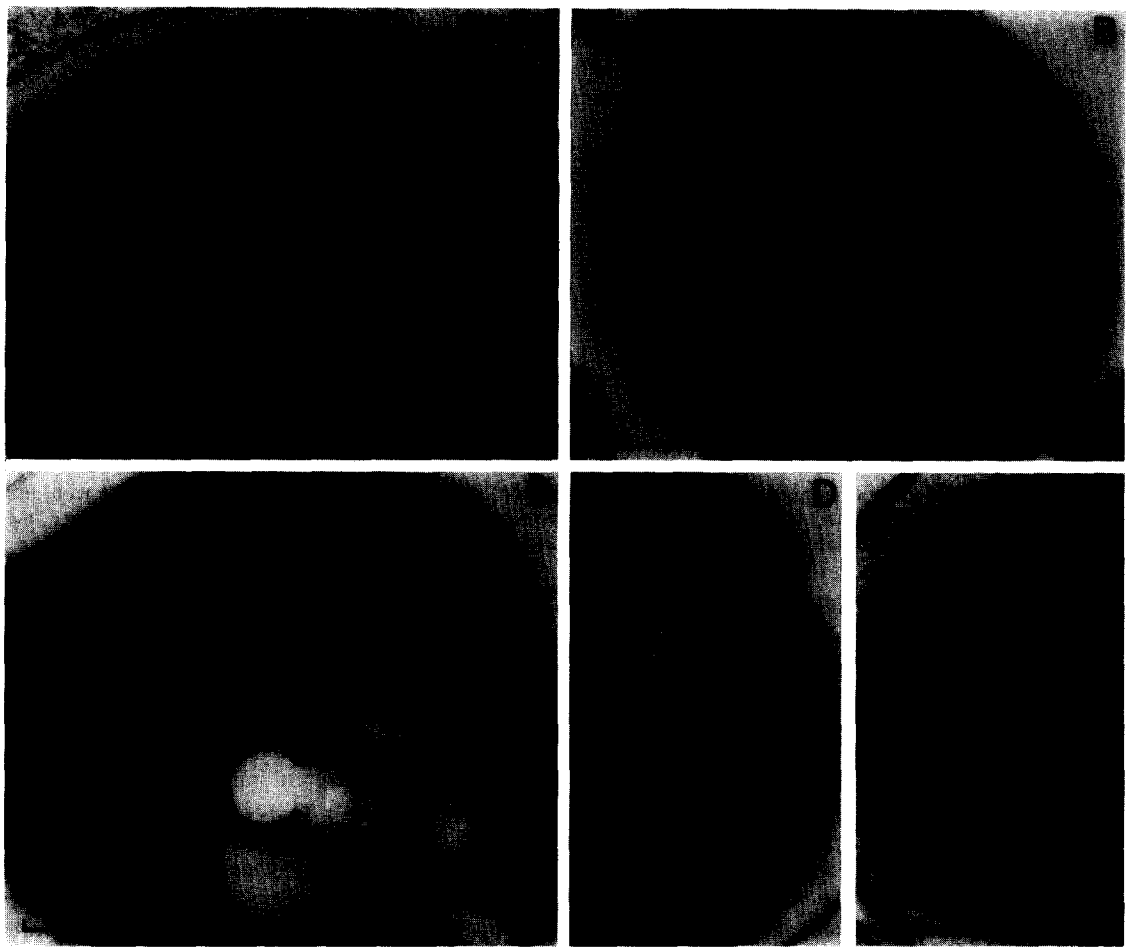

Fig. 3. (A,B) Disappearance of the membranous vesicles in Δ*per4*·P_{AOX}*PER8* cells, shifted from derepressing growth conditions (A) to glucose (B: after 30 min of incubation on glucose; A,B: $KMnO_4$). In identically grown cells of Δ*per10*·P_{AOX}*PER8* (C) this disappearance was not observed and the vesicles were virtually unaffected (D). (E) Shows the presence of vesicles in derepressed cells of Δ*per5*·P_{AOX}*PER8* (C–E: labeled with anti-Per8p antibodies). *, alcohol oxidase crystalloid; N, nucleus. The bar represents 0.5 μm.

4. Discussion

We have analyzed the presence of peroxisomal membrane remnants in three peroxisome-deficient (*per*) mutants of the yeast *Hansenula polymorpha*. The existence of such structures in yeast peroxisome-deficient mutants has been a matter of debate since they were apparently evident, e.g. in *H. polymorpha per1* [15], in bakers yeast *peb2* and *peb4* [6] and several *Pichia pastoris pas* mutants [16,17] as well as in higher eukaryotic cells [5] but, on the other hand, still undetectable in various other baker's yeast *pas* mutants and *H. polymorpha* Δ*per9* [7] and ts6 [18].

In fully derepressed *PER4, PER5* and *PER10* disruptants membranous vesicles were readily detectable which were never detected in glucose-grown cells. This inducibility, together with their architecture in freeze-etch replicas and the fact that they are the target for overproduced Per8p, strongly suggest that they indeed represent peroxisomal membrane remnant in peroxisome-deficient *H. polymorpha* cells.

As reported previously [15], it was difficult to demonstrate unequivocally peroxisomal vesicles in *H. polymorpha per* disruption strains (Δ*per*), although peroxisomal membrane proteins (PMPs) were normally synthesized at wild-type (WT) levels in the various strains. However, the actual level of these PMPs is very low, even in fully derepressed cells, which generally prevents their identification by EM methods, while the low numbers of vesicles did not allow their biochemical characterization after conventional cell fractionation procedures. For their purification, we took advantage of the previously described *H. polymorpha* peroxisome proliferation factor (Per8p [9]) which also appeared to be effective in the multiplication of the peroxisomal remnants in the *per* deletion strains, used in this study. The use of Per8p had two advantages: it proved to be useful as a tag for the identification of peroxisomal vesicles in ultrastructural studies and also allowed the isolation of these structures by density centrifugation.

However, it must be emphasized that the use of endogenous PMP markers may lead to undesired side-effects. For instance, overproduction of *H. polymorpha* Per9p in either the WT or a Δ*per9* strain results in a Per^- phenotype, typified by mislocation of peroxisomal matrix enzymes in the cytosol and the accumulation of numerous small vesicles characterized by the presence of Per9p [7]. A comparable result was obtained after overproduction of Per10p [8]. We assume that these vesicles cannot be considered 'authentic' ghosts but instead arise due to the disturbance of the protein import machinery as a result of overexpression of Per9p or Per10p. Thus, Per9p and Per10p (and hence most probably also their heterologous counterparts, e.g. *S. cerevisiae* Pas3p) may not be suitable

candidates for tagging of peroxisomal remnants unless their synthesis is accurately controlled or, perhaps, the proteins are functionally inactivated. In this context less harm is expected from *H. polymorpha* Per8p since overproduction in WT cells leads to the proliferation of numerous intact, functional and protein import-competent peroxisomes [9]. Our data strongly suggest that the vesicles in the three Δ*per* strains reacted like intact peroxisomes upon Per8p overproduction and proliferated.

In summary:

- Peroxisomal vesicles have been demonstrated in *H. polymorpha* Δ*per4*, Δ*per5* and Δ*per10* cells.
- They do not contain major matrix proteins. The presence of a small amount of AO protein in the purified fractions is explained as adhesion to the vesicular membranes during the isolation procedure. This is a common observation for purified peroxisomal membranes from *H. polymorpha* and has also been observed for other matrix proteins like *H. polymorpha* D-amino acid oxidase (Sulter and Veenhuis, unpublished results) and *Candida boidinii* PMP20 [19], which behave like integral PMPs based on biochemical criteria.
- The peroxisomal vesicles display typical peroxisomal properties in that (i) they are inducible in nature, (ii) proliferate upon overexpression of Per8p, the *H. polymorpha* peroxisome proliferation factor, and (iii) may be susceptible to carbon-catabolite inactivation.

Other properties, which are attributed to normal intact peroxisomes, may also be maintained in the peroxisomal vesicles depending on the specific deletion strain in which they occur. In order to obtain further clues as to their properties, we are currently analyzing the protein composition of the Per8p-tagged vesicles from selected *PER*-deletion strains in depth. Where conventional fractionation does not give unequivocal results, we now apply an immuno-magnetic sorting procedure, using magnetic beads coated with anti-Per8p antibodies. The initial analysis of these fractions revealed that the vesicles also contain, next to Per8p, other peroxisomal matrix and membrane proteins (e.g. Per3p and Per10p; Van der Klei et al., unpublished results).

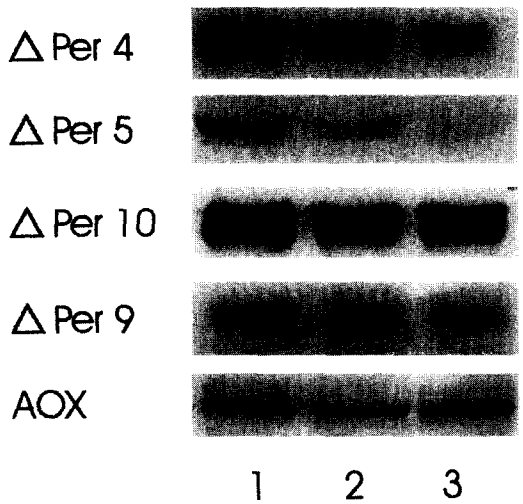

Fig. 4. Western blots of derepressed cells of Δ*per4*·P_{AOX}*PER8*, Δ*per5*·P_{AOX}*PER8* and Δ*per10*·P_{AOX}*PER8* (lane 1), showing the degradation of Per8p in cells of Δ*per4* and Δ*per5*, but not Δ*per10*, after incubation of the cells for 1 (lane 2) and 2 h (lane 3) in the presence of glucose. In cells of Δ*per9*·P_{AOX}*PER8*, which do not contain membrane vesicles and are taken as a control, this degradation of Per8p was not observed. Also, cytoplasmic AO, present in Δ*per4*·P_{AOX}*PER8* cells, remained unaffected (lower panel anti-alcohol oxidase). Equal amounts of protein were loaded per lane. Blots were decorated using anti-Per8p antibodies unless indicated otherwise.

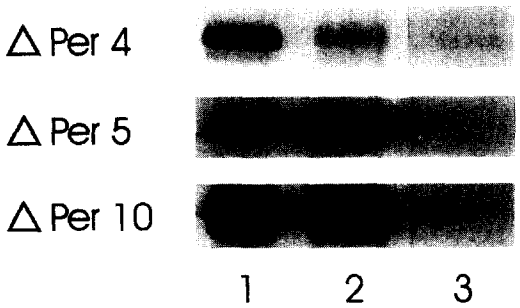

Fig. 5. Western blots of organellar fraction obtained after differential centrifugation of homogenized protoplasts prepared from derepressed cells of Δ*per4*·P_{AOX}*PER8*, Δ*per5*·P_{AOX}*PER8* and Δ*per10*·P_{AOX}*PER8* to demonstrate the sedimentable nature of Per8p, produced in these constructed mutants. (Lane 1) 30 000×pellet, (lane 2) 100 000×*g* pellet of the 30 000×*g* supernatant, (lane 3) 100 000×*g* supernatant. Equal amounts of protein were loaded per lane. The blots were decorated with anti-Per8p antibodies.

The fact that the vesicles in Δ*per10* strains were not degraded under glucose-excess conditions indicates that Per10p is not only essential for protein import [8], but may also play a role in the peroxisome degradation pathway [20]. This result again stresses the functional relationship which exists between various gene products involved in peroxisome biogenesis and maintenance in *H. polymorpha* [21].

Based on our data we propose that the term 'peroxisomal ghosts' should only be used to indicate the peroxisomal membrane vesicles which remain in peroxisome-deficient mutants and are characterized by the presence of various PMPs but devoid of major matrix proteins ('empty vesicles'; this paper [5]). The organelles remaining in other cases, e.g. in mutants blocked in import of PTS1 or PTS2 proteins, can be considered imperfect (e.g. *Pichia pastoris pas8, S. cerevisiae pas7*).

The possibility that peroxisomal ghosts may regain their protein import capacity or alternatively, that only a subset of ghosts is capable to import, as for peroxisomes in WT cells, after re-introduction of the deleted *PER* gene, is currently being explored in our laboratory.

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