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Peroxisome biogenesis in *Hansenula polymorpha*: different mutations in genes, essential for peroxisome biogenesis, cause different peroxisomal mutant phenotypes

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1. SUMMARY

In *Hansenula polymorpha*, different monogenic recessive mutations mapped in either of two previously identified genes, *PER1* and *PER3*, produced different peroxisomal mutant phenotypes. Among five *per1* mutants, four showed a Pim⁻ phenotype: the cells contained few small peroxisomes while the bulk of the matrix enzymes resided in the cytosol. One of these mutants, *per1-124* had an enhanced rate of peroxisome proliferation. The fifth mutant completely lacked peroxisomes (Per⁻ phenotype). Of seven *per3* mutants, four displayed a Pim⁻ phenotype, two others a Per⁻ phenotype, while one mutant showed pH-dependent growth on methanol and was affected in oligomerization of peroxisomal matrix protein. Thus, the protein products of both *PER1* and *PER3* genes appear to be essential in different aspects of peroxisome assembly/proliferation.

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2. INTRODUCTION

Microbodies (peroxisomes) represent the most recently discovered class of subcellular organelles. They are ubiquitously present in eukaryotic cells [1,2] and may be involved in a variety of metabolic processes, which are partly inducible in nature [1–4]. This functional diversity has given strong impetus to current research aimed at elucidating the molecular mechanisms involved in microbody biogenesis and function.

Yeasts are attractive model organisms for such studies since the proliferation and enzymic composition of peroxisomes in these organisms can be readily manipulated by varying growth conditions [3]. In recent years, a series of mutants impaired in peroxisome biogenesis/function have been isolated from various organisms, including yeasts, and several of the corresponding genes are cloned

and sequenced [5–13]. Analysis of genes essential for peroxisome biogenesis in *Saccharomyces cerevisiae* [5–9] stressed the complexity of the genetic system controlling microbody biogenesis and the diversity in function and location of components recquired for the proliferation and assembly of intact, functional organelles.

Recently, we have identified several genes essential for peroxisome biogenesis and assembly in the methylotrophic yeast *Hansenula polymorpha* and drawn attention to a high degree of integrity in their functions [10,11]. Here, we report that different mutations in any of two previously identified genes, *PER1* and *PER3* [11], lead to different phenotypes varying from the complete absence of peroxisomal structures to specific defects in peroxisomal protein import and effects on organelle proliferation and assembly. This is the first report on the multiplicity in functions of components involved in peroxisome biogenesis and assembly in yeast.

3. MATERIALS AND METHODS

3.1. Microorganisms and growth conditions

All mutants were derived from wild-type *Hansenula polymorpha* CBS 4732 [10]. Different, multiple backcrossed representatives of complementation groups *PER1* and *PER3* [11] were grown in chemostat cultures ($D = 0.07$ h^{-1}) at 37°C in mineral medium [14] on mixtures of glucose (0.25%, w/v)/methanol (0.2%, v/v) [15].

3.2. Mutant isolation and genetic methods

The mutagenesis by NTG treatment and subsequent isolation of peroxisome assembly mutants has already been described [10]. Initial mutants were backcrossed four times with auxotrophic mutants of *H. polymorpha* NCYC 495 (*ade11-1, met6-1, ade11-1 met6-1 and leu1-1*; [16]) prior to genetic characterization. Mating, complementation analysis, sporulation, random spore analysis and linkage analysis were performed as described [10,11,16].

3.3. Electron microscopy

Whole cells were fixed with 1.5% (w/v) $KMnO_4$ for 20 min at room temperature. Spheroplasts, prepared by the method of Douma et al. [17], were pre-fixed in 6% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 60 min at 0°C, followed by post-fixation in a mixture of 0.5% (w/v) OsO_4 and 2.5% (w/v) $K_2Cr_2O_7$ in the cacodylate buffer for 90 min at 0°C. The samples were embedded in Epon 812; ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

Immunocytochemistry was performed on sections of (i) intact cells, fixed in glutaraldehyde and embedded in Lowicryl K4M [17]; or (ii) spheroplasts, fixed and embedded in Epon as described above; the latter sections were bleached with H_2O_2 prior to labelling. Immunolabelling was performed by the protein A/gold method, using specific antibodies against alcohol oxidase [17].

4. RESULTS AND DISCUSSION

Recently, we have shown that several monogenic recessive mutations mapped in five different genes, designated *PER1–PER5*, caused defects in peroxisomal matrix protein import (Pim$^-$ phenotype) in the methylotrophic yeast *H. polymorpha* [11]. The Pim$^-$ mutants described were characterized by the inability to grow on methanol

Table 1

Linkage data obtained after random spore analysis of *per1* and *per3* mutants of *H. polymorpha*

Pair of alleles	Observed number of segregants		Linkage
	Parentals	Recombinations	
per1-124 / per1-157	734	0	Yes
per1-124 / per1-201	582	0	Yes
per1-124 / per1-230	911	0	Yes
per1-124 / per1-172	1037	0	Yes
per3-188 / per3-191	803	0	Yes
per3-188 / per3-229	536	0	Yes
per3-188 / per3-237	487	0	Yes
per3-188 / per3-162	2081	6	Yes
per3-188 / per3-220	1847	5	Yes
per3-188 / per3-101	967	0	Yes
per1-124 / per3-188	197	206	No

(methanol utilization-defective; Mut$^-$) and, morphologically, by the presence of few small intact peroxisomes, while the bulk of the peroxisomal matrix proteins resided in the cytosol. In addition, a collection of Per$^-$ mutants, characterized by the complete absence of peroxisomes [10,11],

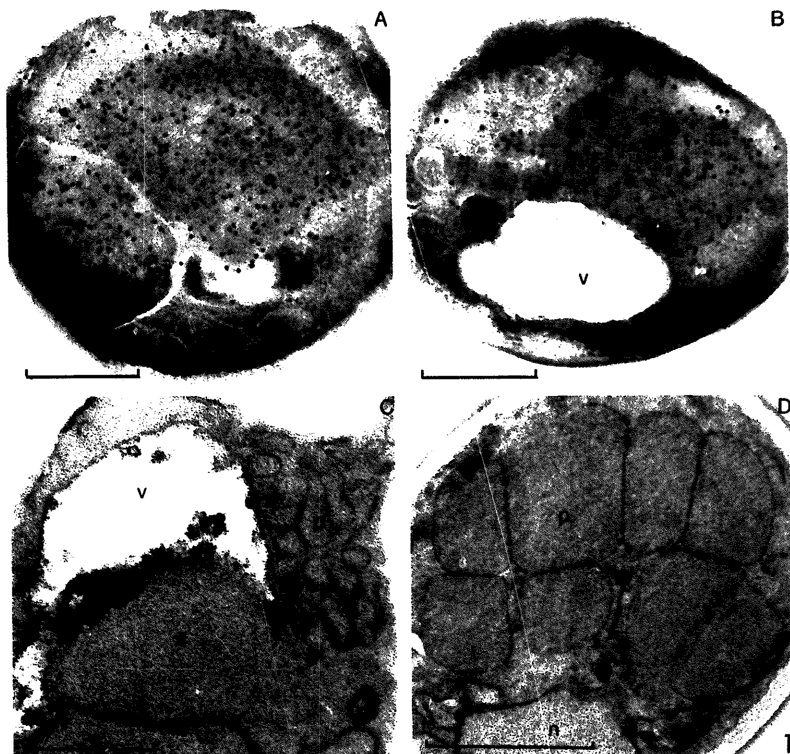

Fig. 1. Micrographs were taken of intact cells, fixed with $KMnO_4$, unless otherwise indicated. Bars = 0.5 μm. Abbreviations: N, nucleus; M, mitochondrion; V, vacuole; P, peroxisome. (**A**, **B**) (Glutaraldehyde Lowicryl, uranyl acetate). Labelling patterns of alcohol oxidase protein after immunocytochemical experiments on sections of cells of *per1-172*, completely lacking peroxisomes (**A** Per$^-$ phenotype) and *per1-230*, which still contains few small peroxisomes while the bulk of the alcohol oxidase is on the cytosolic crystalloid (**B**, Pim$^-$ phenotype). (**C**) Detail of a cell from mutant *per1-124*, showing numerous small peroxisomes; the AO crystalloid, which is poorly preserved after $KMnO_4$ fixation, is indicated by an asterisk. (**D**) Survey of a cell of mutant *per3-101*, grown at pH 7.0, showing normal peroxisomal proliferation.

were isolated. In this study we focussed on the characterization of additional mutants allocated either to complementation group *PER1* (five strains) or *PER3* (seven strains). The different representatives of both groups were subjected to detailed genetic and morphological analyses. Genetic analysis of backcrossed mutant strains indicated that each of the 12 mutant phenotypes was determined by a single recessive nuclear mutation.

The linkage analysis confirmed that the five independently isolated mutations are located in gene *PER1* (Table 1). Morphological analysis of these mutants, including analysis of series of serial sections and immunocytochemistry, revealed that the above mutations, mapped in the *PER1* gene, may cause three different defects in peroxisome assembly and proliferation. Mutant alleles *per1-157, 201, 230* and *124* caused a morphologically identical Pim$^-$ phenotype: fully derepressed cells (from glucose/methanol-grown chemostat cultures) contained small peroxisomes, while the bulk of the peroxisomal matrix proteins was mislocalized to the cytosol and the nucleus (Fig. 1B). Moreover, mutant allele *per1-124* (Fig. 1C) caused a significant increase in the average number of peroxisomes (up to 5–10 times greater than those in the wild-type [11]); *per1-172*, however, caused complete absence of morphologically recognizable peroxisomal structures (Fig. 1A; Per$^-$ phenotype).

Seven mutations mapped in the *PER3* gene (Table 1) also determined different abnormalities in peroxisome biogenesis and assembly. Four mutants, namely *per3-188, 191, 229* and *237* were morphologically identical and showed the 'normal' Pim$^-$ phenotype, described above (compare Fig. 1B). Two other mutants, *per3-162* and *220* completely lacked peroxisomes and thus displayed a Per$^-$ phenotype (compare Fig. 1A). A

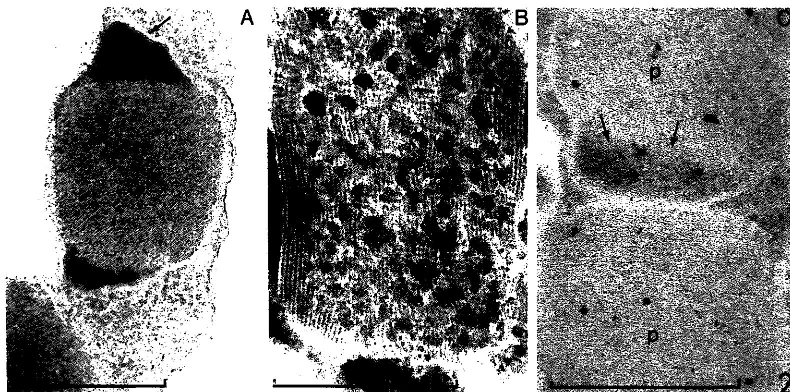

Fig. 2. Micrographs were taken of intact cells, fixed with $KMnO_4$, unless otherwise indicated. Bars = 0.5 μm. Abbreviations as in Fig. 1. (**A**) Typical electron dense inclusions (arrow) in the crystalline peroxisomal matrix of mutant *per3-101*, 8 h after the shift from pH 5.0 to pH 7.0. In steady-state cells from chemostat cultures on glucose/methanol (**B**) these inclusions are distributed throughout the peroxisomal matrix (**A**, **B**; spheroplasts, glutaraldehyde, OsO_4). (**C**) Results of an immunocytochemical experiment in which sections of Epon-embedded cells, shown in Fig. 2A, are bleached with H_2O_2, and subsequently labelled with antibodies against alcohol oxidase and protein A/gold. In spite of the low-labelling intensities obtained at these conditions, labelling of the 'aggregated' part of the peroxisomal part of the matrix is evident.

third phenotype was observed in mutant *per3-101*. Growth of this mutant on methanol was pH-sensitive; normal methylotrophic growth, at rates observed for the wild-type, occurred at pH 5.0; however, growth on methanol was fully impaired at pH 7.0 [18]. Morphologically, intact peroxisomes fully identical to those in wild-type cells were present in mutant cells grown on methanol at pH 5.0. In cells incubated at pH 7.0, the size and number of peroxisomes was not affected (Fig. 1D). However, drastic alterations in the crystalline substructure of the organellar matrix were observed after a shift of cells from pH 5.0 to pH 7.0. In thin sections of glutaraldehyde/OsO_4-fixed spheroplasts, prepared from cells in the initial hours after the pH shift, electron-dense inclusions were seen (Fig. 2A); these became randomly distributed throughout the crystalline matrix of organelles present in steady state cells (Fig. 2B). FPLC analysis of crude extract prepared from cells grown in chemostat cultures on glucose/methanol mixtures at pH 7.0 indicated the presence of monomers of alcohol oxidase, which were not detected in cells grown at pH 5.0 (5.3. van der Klei, unpublished data). After immunocytochemical experiments, using specific antibodies against alcohol oxidase and protein A/gold, the electron dense inclusions were positively labelled (Fig. 2C), indicating that they most probably represent inproperly assembled alcohol oxidase protein. However, in contrast to the other *per3* strains, a cytosolic location of alcohol oxidase was never observed. Therefore, in mutant *per3-101* alcohol oxidase assembly, not import, appeared to be affected.

The functional roles of the protein products of the two *PER* genes are still unknown. Detailed analysis of five Pim$^-$ mutants [11] suggested that their corresponding genes encode components of a general import machinery for peroxisomal matrix proteins. At least two explanations may be offered as to why mutations in either *PER1* or *PER3* cause different defects in peroxisome biogenesis and assembly in *H. polymorpha*. Firstly, protein products encoded by these genes are probably multifunctional components that have a role in different aspects of peroxisome biogenesis, such as import and assembly of matrix proteins and peroxisome proliferation. A similar model has been proposed to explain defects in both sorting from Golgi to vacuole and vacuole segregation caused by *vac1* mutations [19]. Secondly, it is shown that multi-subunit protein complexes are involved in protein import and assembly as well as proliferation and function of various other organelles, e.g. yeast mitochondria [20], endoplasmic reticulum [21], Golgi apparatus [22], vacuoles [23] and nuclei [24]. However, functional multiplicity has not yet been observed for different gene products essential for peroxisome biogenesis and proliferation in *S. cerevisiae* [5,6]. However, recently we have found that numerous genes, involved in peroxisome biogenesis and assembly in *H. polymorpha*, including *PER1* and *PER3*, show a high degree of genetic interaction, suggesting functional and physical links between the corresponding gene products (Titorenko et al., submitted to Proc. Natl. Acad. Sci. USA). Conceivably, different mutations in *PER1* and *PER3* can disturb proper functioning of different interacting proteins. An identical mechanism is suggested to explain defects in both vacuolar sorting and segregation, as well as in assembly of the vacuolar H^+-ATPase, caused by *vps3* mutations [25].

We have now cloned the *PER1* and *PER3* genes (H.R. Waterham and P. Haima, unpublished data). Characterization of the gene products will provide further insight in the observed phenomena.

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