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Tan, Xuqiu; Titorenko, Vladimir I.; Klei, Ida J. van der; Sulter, Grietje J.; Haima, Peter; Waterham, Hans R.; Evers, Melchior; Harder, Willem; Veenhuis, Marten; Cregg, James M.

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ORIGINAL PAPER

Xuqiu Tan · Vladimir I. Titorenko
Ida J. van der Klei · Grietje J. Sulter · Peter Haima
Hans R. Waterham · Melchior Evers
Wim Harder · Marten Veenhuis · James M. Cregg

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Abstract In the methylotrophic yeast *Hansenula polymorpha*, approximately 25% of all methanol-utilization-defective (Mut^-) mutants are affected in genes required for peroxisome biogenesis (*PER* genes). Previously, we reported that one group of *per* mutants, termed Pim^- , are characterized by the presence of a few small peroxisomes with the bulk of peroxisomal enzymes located in the cytosol. Here, we describe a second major group of *per* mutants that were observed to be devoid of any peroxisome-like structure (Per^-). In each Per^- mutant, the peroxisomal methanol-pathway enzymes alcohol oxidase, catalase and dihydroxyacetone synthase were present and active but located in the cytosol. Together, the Pim^- and Per^- mutant collections involved mutations in 14 different *PER* genes. Two of the genes, *PER5* and *PER7*, were represented by both dominant-negative and recessive alleles. Diploids resulting from crosses of dominant *per* strains and wild-type *H. polymorpha* were Mut^- and harbored peroxisomes with abnormal morphology. This is the first report of dominant-negative mutations affecting peroxisome biogenesis.

Key words *Hansenula polymorpha* · Peroxisomes · Organelle biogenesis

Introduction

Eukaryotic cells are elaborately subdivided into functionally distinct membrane-bound compartments or organelles. Peroxisomes represent a unique class of such organelles present in virtually all eukaryotes (Lazarow and Fujiki 1985; Borst 1989; Subramani 1993). Peroxisomal-matrix enzymes are involved in a number of important metabolic processes but vary depending on the organism, tissue and environmental conditions. In humans, peroxisomes play an essential role in intermediary cellular metabolism as demonstrated by the existence of a family of lethal genetic disorders, referred to as Zellweger syndrome, in which the peroxisomes are defective (Lazarow and Moser 1989).

It is now generally accepted that peroxisomes form by budding or dividing from pre-existing peroxisomes (Veenhuis et al. 1978). Peroxisomal proteins are encoded by nuclear genes, synthesized on free ribosomes, and imported post-translationally into the organelles in an ATP-dependent manner (Fujiki et al. 1984; Imanaka et al. 1987; Wendland and Subramani 1993). Many peroxisomal-matrix proteins are imported via a tripeptide targeting signal (SKL or conservative variants) located at the C-terminus (Gould et al. 1987). This peroxisomal targeting signal (PTS) is both necessary and sufficient to direct proteins into the organelle, and is evolutionarily conserved (Gould et al. 1989, 1990; Keller et al. 1991). Recent studies have revealed the existence of a second PTS which is present at the N-terminus of a few peroxisomal proteins (Osumi et al. 1991; Swinkels et al. 1991). Additional PTS systems may also exist (de Hoop and AB 1992). Aside from PTSs, general features of the molecular import machinery have yet to be elucidated.

Genetics has only recently been applied as an experimental tool to investigate peroxisomes (Lazarow 1993). In fact, the first identified mutants affected in peroxisome function were human Zellweger patients

X. Tan · J.M. Cregg (✉)
Department of Chemistry, Biochemistry, and Molecular Biology,
Oregon Graduate Institute of Science and Technology, Portland,
OR 97291-1000, USA

V.I. Titorenko · I.J. van der Klei · G.J. Sulter · P. Haima
H.R. Waterman · M. Evers · W. Harder · M. Veenhuis (✉)
Laboratory for Electron Microscopy, Biological Center, State
University of Groningen, 9751 NN Haren, The Netherlands

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(Goldfischer et al. 1973). Cells from such patients are deficient in peroxisomes and, as a result, most peroxisomal enzymes are mislocalized to the cytosol (Lazarow and Moser 1989). Evidence suggests that the deficiency is a consequence of defects in a major matrix-protein import mechanism (Santos et al. 1988 a,b; Walton et al. 1992). Somatic cell-fusion studies indicate that mutations in at least eight different genes are responsible for the disorder (Brul et al. 1988; Roscher et al. 1989). In addition to humans, peroxisome-deficient mutants have also been reported in Chinese hamster ovary cell lines (Zoeller and Raetz 1986; Tsukamoto et al. 1990) and in four yeast species: *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Pichia pastoris*, and *Yarrowia lipolytica*.

These yeast species are attractive model systems for peroxisome studies since the proliferation and enzymic composition of peroxisomes can be readily manipulated by varying the growth conditions of the yeasts (Veenhuis and Harder 1991). Peroxisomes are typically small and few in number in cells of these yeasts cultured on media with a carbon source, such as glucose, but large and numerous when cultured on methanol or oleate medium. In addition, peroxisome-deficient yeast strains are ideal conditionally lethal mutants in that they are healthy in glucose medium but non-viable only in methanol and/or oleate medium. To-date, peroxisome-deficient mutants affected in 15 genes have been described in *S. cerevisiae* (Erdmann et al. 1989; Kunau and Hartig 1992; Elgersma et al. 1993; Zhang et al. 1993), in 14 genes in *H. polymorpha* (Cregg et al. 1990; Didion and Roggenkamp 1990; Waterham et al. 1992; Titorenko et al. 1993; this report), in ten genes in *P. pastoris* (Liu et al. 1992; Gould et al. 1992), and in two genes in *Y. lipolytica* (Nuttley et al. 1993). In all these peroxisome-deficient yeast mutants, peroxisomal-matrix enzymes are synthesized but, with two notable exceptions, are mislocalized to the cytosol. The exceptions are *P. pastoris pas8* mutants (*S. cerevisiae pas10*, *H. polymorpha per3*), in which PTS1 enzymes are located in the cytosol but the PTS2-enzyme thiolase is present in small peroxisomes, and *S. cerevisiae pas7* mutants, in which PTS1 enzymes, but not thiolase, are imported normally (McCollum et al. 1993; van der Leij et al. 1993; Marzioch et al. 1994; unpublished results). Evidence suggests that the PAS8 product is the receptor for PTS1 proteins while the PAS7 product may be the PTS2-protein receptor.

The methylotrophic yeast *H. polymorpha* is especially attractive for genetic studies on peroxisome biogenesis (Veenhuis 1992). In this organism, detailed biochemical and physiological information exists on the role of peroxisomes in the metabolism of certain substrates including carbon sources, such as methanol and ethanol, and nitrogen sources, such as primary amines, D-alanine and uric acid (Veenhuis and Harder 1987 1991). In addition, methods for classical- and molecular-genetic manipulation of the organism are

well developed (Cregg 1987; Gleeson and Sudbery 1988; Faber et al. 1992; Titorenko et al. 1993).

Previously, we described the isolation of two peroxisome-deficient mutants, termed *per* mutants, from a collection of strains defective in their ability to grow on methanol (*Mut⁻*) (Cregg et al. 1990). These mutants were identified by direct electron microscopic observation of methanol-induced cells. Continued screening of the *Mut⁻* collection has resulted in the identification of an additional 58 *per* mutants. Based on peroxisome appearance in methanol-induced mutant cells, three distinct phenotypic classes could be distinguished. In the first class, peroxisomes are completely absent (*Per⁻* phenotype). In the second class, peroxisomes are present but abnormally small in size (*Pim⁻* phenotype). Recent studies on the *Pim⁻* mutants revealed that the bulk of peroxisomal enzymes are mislocalized to the cytosol in these mutants (Waterham et al. 1992). The third phenotypic class of *per* mutants induces peroxisomes that are normal in size and number but harbor an aberrant crystalline matrix substructure (*Pss⁻* phenotype) (Titorenko et al. 1993). In this report, we describe the identification and characterization of the *Per⁻* collection.

Materials and methods

Strains, media and growth conditions. *H. polymorpha* strains used in this study are listed in Table 1. Cells were grown in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in YNB minimal medium (0.67% yeast nitrogen base without amino acids) supplemented with 0.4% glucose. For growth on methanol, YNB minimal medium was prepared with 0.05% yeast extract and 0.5% methanol. Nutritional supplements for the support of auxotrophic growth (methionine, leucine, adenine, arginine, or uracil and uridine) were added to 50 µg/ml as required. Sporulation (mating) medium was composed of 2% malt extract. Methanol-induced cells were prepared by pre-culturing strains two times in YPD medium for approximately 24 h each, then shifting cultures to methanol by the addition of one part of the second YPD culture to three parts of YNB medium with 0.5% methanol. Except where indicated in the text, all growth was at 37 °C.

Genetic analysis. Genetic procedures, such as mutagenesis, *per* mutant screening, mating, random spore analysis and backcrossing, were performed as previously described (Gleeson and Sudbery 1988). The standard procedure for complementation testing was modified to effect a separation of mating and complementation phases. Each *per* mutant was first crossed with each of two different auxotrophically marked strains (usually *leu1* and *ade11* mutants but occasionally *met6*, *ura3* or *arg2*), and two sets of marked *per* spore products were isolated. One set of marked *per* strains was patched onto YPD agar plates (approximately 12 patches per plate) and the other marked set was spread as a lawn onto a series of YPD plates. The plates were then incubated overnight at 37 °C. Next, cells from paired sets of patched plates and lawn plates were transferred by the replica-plating technique onto plates containing sporulation medium to initiate mating. After incubation at 37 °C for 1–2 days, cells were further transferred to YNB minimal glucose plates to allow for the selective growth of prototrophic diploid cells and to prevent the growth of non-mated or self-mated cells. After incubation for 3 days at 37 °C, the resulting prototrophic diploid colonies were spread on

Table 1. *H. polymorpha* strains

Strain	Genotype	No. of alleles ^a	Reference
CBS4732	Wild-type		CBS4732
A16	<i>leu1</i>		Veale et al. (1992)
MH122-3	<i>ura3</i>		Gleeson and Sudbery (1988)
MH120-1	<i>met6</i>		Gleeson and Sudbery (1988)
MH119-1	<i>ade11</i>		Gleeson and Sudbery (1988)
MH100-2	<i>arg2</i>		Gleeson and Sudbery (1988)
C36	<i>per1</i>	1 (11)	This study
C27	<i>per2</i>	1 (4)	Cregg et al. (1990)
C26	<i>per3</i>	3 (7)	This study
C16	<i>per4</i>	1 (4)	Waterham et al. (1992)
C79	<i>per5</i>	2	Waterham et al. (1992)
C4	<i>PER5^D</i>	1	This study
C72	<i>per6</i>	1 (2)	Titorenko et al. (1993)
C111	<i>per7</i>	1 (10)	This study
C35	<i>PER7^D</i>	1	This study
C76	<i>per8</i>	3	This study
B16	<i>per9</i>	2	This study
B64	<i>per10</i>	2	This study
C60	<i>per11</i>	1	This study
3P	<i>per12</i>	1	This study
C139	<i>per13</i>	2	This study
C88	<i>per14</i>	1	This study
C4/wt	<i>PER5^D/PER5</i>		This study
C35/wt	<i>PER7^D/PER7</i>		This study

^a Number of allelic strains used in this study. Parentheses indicate the total number of *per* alleles including those previously described (Cregg et al. 1990; Waterham et al. 1992; Titorenko et al. 1993)

YNB minimal methanol plates to test for a Mut phenotype. Genetic mapping experiments were performed as described by Waterham et al. (1992).

Cellular fractionation. Mut⁺ strains were pre-cultured in YPD medium to a OD₆₀₀ of approximately 0.75 and then shifted by centrifugation into 1 l of YNB methanol medium at a starting OD₆₀₀ of 0.005. Mut⁻ strains were pre-cultured in 1 l of YPD medium and the entire culture was shifted at an OD₆₀₀ of approximately 0.75 into 1 l of methanol medium. Cultures were incubated for 16 h and harvested by centrifugation. Cells were washed three times with 10 ml of distilled water and resuspended in 4 ml of digestion buffer [5 mM K⁺ 3-(N-morpholino) propanesulphonate (pH 7.2), 0.5 M KCl, 10 mM Na₂SO₃] per gram of cells. Cells were then converted to protoplasts by the addition of Zymolyase 100T (ICN, Costa Mesa, Calif.) to 0.25 mg/ml and incubated at 37°C for 30–40 min with occasional shaking. All subsequent steps were carried out at 4°C. Protoplasts were diluted with 10 vol of ice-cold 1 M sorbitol and centrifuged at 4000 g for 10 min. The resulting pellets were gently resuspended in sorbitol-MES buffer [1 M sorbitol, 5 mM K⁺ 2-(N-morpholino)ethanesulfonic acid, pH 5.5] at 1 ml per gram of cells. An equal volume of 0.25 M sorbitol, 5 mM MES (pH 5.5) was then added to the suspension while gently shaking and the mixtures were placed on ice for 10 min. A volume of 1.75 M sorbitol, 5 mM MES (pH 5.5) was then added sufficient to bring the solution back to 1 M sorbitol and samples were centrifuged at 1000 g for 20 min to remove unbroken cells and other debris. The supernatants were subjected to centrifugation at 25 000 g for 25 min and the resulting pellets were resuspended in 100 µl of sorbitol-MES buffer and, along with supernatant fractions, were examined for enzyme activities.

Other methods. Electron microscopy and immunocytochemistry were performed as described previously (Waterham et al. 1992). Immunofluorescence experiments were carried out using rabbit polyclonal antibodies against total peroxisomal integral membrane proteins of *H. polymorpha* and fluorescein isothiocyanate (FITC)-labeled anti-rabbit antibodies (Sulter et al. 1993 a). Total protein in samples was measured by the method of Bradford (1976) with

bovine serum albumin as a standard. AOX (van der Klei et al. 1990), catalase (Ueda et al. 1990), and fumarase (Tolbert 1974) activities were measured according to published procedures.

Results

Identification of peroxisome-deficient mutants

In a previous report, we established that mutants with defects in peroxisome biogenesis exist as a subset within collections of Mut⁻ mutants of *H. polymorpha* (Cregg et al. 1990). Based on this observation, we screened a collection of 260 Mut⁻ strains by electron microscopy (EM) for mutants with defects in peroxisome morphology. Cells of each strain were induced in methanol medium and prepared for EM analysis. Under these conditions, ultra-thin sections from wild-type *H. polymorpha* and most Mut⁻ strains showed virtually normal peroxisome proliferation (Fig. 1 A). However, 60 of the mutant strains had peroxisomal abnormalities (*per* mutants). The majority (38) of these were devoid of any recognizable peroxisome structures (Fig. 1 B) and this phenotypic class of *per* mutants was named Per⁻. As indicated in the Introduction, two additional *per* mutant peroxisome phenotypes, Pim⁻ and Pss⁻, were also observed (reviewed in Veenhuis 1992).

Genetic analyses

Each of the 38 Per⁻ strains was crossed with one or more auxotrophically marked Mut⁺ strains and

Fig. 1 Ultrathin sections of a methanol-induced Per^+ (Mut^-) strain (A) and a Per^- strain (per2) (B) of *H. polymorpha*. Normal peroxisomes are observed in the Per^+ cell but are absent in the Per^- cell. Instead the cell contains a cytoplasmic AOX crystalloid. The structure of the crystalloid is poorly preserved due to KMnO_4 -fixation. Abbreviations: M mitochondrion; N nucleus; P peroxisome; V vacuole. Bar, 1 μm

diploids were selected on minimal methanol medium. Crosses involving 36 of the mutants yielded prototrophic Mut^+ diploid strains with normal-appearing peroxisomes (Per^+) (analysis of the remaining two *per* mutants is described in the last section of the Results.) The resulting diploids were sporulated and random analysis of spore populations revealed a 1:1 ratio of Mut^- and Mut^+ phenotypes from each. Selected spore products were further examined for peroxisomes and in each case peroxisome deficiency co-segregated with Mut^- and vice versa. Thus, each of the 36 mutants appeared to be the consequence of a single recessive nuclear mutation.

The 36 *per* strains were each subjected to complementation analysis. To aid in the performance of this analysis, two sets of oppositely marked auxotrophic derivatives of each *per* strain were first constructed. The two sets of *per* strains were then crossed against each other and prototrophic diploids selected on minimal glucose medium. Diploid colonies were then tested for complementation of *per* alleles by their ability to grow on methanol. Results indicated that the 36 strains represented approximately 12 different complementation groups.

The assignment of mutant alleles to specific genes from the results of complementation testing is occasionally in error due to interallelic complementation between mutant alleles in the same gene or to unlinked non-complementation between mutant alleles of different

genes (Titorenko et al. 1993). To definitively assign *per* alleles to their proper *PER* genes, selected backcrossed *per* mutants were utilized in genetic mapping experiments. For this, each mutant was crossed with each of the other mutants and recombination distances were estimated by the random spore method. Eleven *PER* genes were defined by genetic mapping, one less than our original estimate of 12 due to one case of interallelic complementation between two mutant alleles.

We also crossed a mutant representative of each of the 11 *PER* genes with a mutant representative in each of the five Pim^- groups defined as defective in genes *PER1* through *PER5* and the Pss^- strain in *PER6*. The complementation and mapping results demonstrated that three representatives were alleles of previously defined *PER* genes (*PER1*, *PER3* and *PER5*), while the remaining eight were defective in new *PER* genes which we named *PER7* through *PER14*. Thus, the total *per* mutant collection contained mutations in 14 different *PER* genes (see Table 1). As diagrammed in Fig. 2, the mapping results revealed three linkage groups each of which contained several *PER* genes. The relative order of genes in each group was determined through a series of three-factor crosses. Linkage group 1 included the following genes in order: *MET6*, *PER5*, *PER7*, *PER4* and *PER6*. (*MET6* is a locus previously identified by Gleeson and Sudbery 1988). Linkage group 2 contained the following genes: *PER14*, *PER13*, *PER1*, *PER9* and *PER2*. Linkage group 3 contained the genes: *PER3*, *PER10* and *PER11*. Two genes, *PER8* and *PER12*, were unlinked to each other and to any of the other *PER* genes.

Methanol-induced cells of backcrossed mutant representatives in each *PER* gene were serial sectioned and carefully examined for peroxisomes by EM. Most

Fig. 2 Genetic map of *H. polymorpha* *PER* genes as deduced by random spore analysis. A representative *per* mutant from each *PER* group was crossed with mutant representatives from each of the other groups and the resulting diploids were sporulated. Approximately 1000 spore products from each cross were germinated under non-selective growth conditions and tested for a *Mut* phenotype. Crosses in which less than 25% of spore products were *Mut*⁺ were examined further for potential genetic linkage by backcrossing *Mut*⁻ spore products to their respective parental *per* strains. Recombination distances are reported as the number of non-parental spore products (wild-type + *per* double mutants) divided by the total number of spore products (non-parental + parental) expressed as a percentage. *PER8* and *PER12* are not linked to each other or to any of the other *PER* genes

mutants were still *Per*⁻ including: C36 (*per1*), C79 (*per5*), C111 (*per7*), C76 (*per8*), B16 (*per9*), B64 (*per10*), C60 (*per11*), and 3P (*per12*). However, two strains, C26 (*per3*) and C139 (*per13*) which were *Per*⁻ prior to backcrossing, were *Pim*⁻ after backcrossing, and another, C88 (*per14*), was *Pss*⁻. Thus, it appeared that, prior to backcrossing, these three strains contained secondary mutations that influenced the mutant peroxisome phenotype. Interestingly, two *PER* genes, *PER1* and *PER5*, were represented by both *Per*⁻ and *Pim*⁻ mutant alleles. Thus, it appears that, for these two genes at least, the mutant peroxisome phenotype was allele specific.

Localization of peroxisomal enzymes

Backcrossed mutant representatives in each new *PER* gene (*PER7*–*PER14*) and newly identified *Per*⁻ representatives in *PER1* and *PER5* were examined for the presence, activity and subcellular location of selected peroxisomal enzymes. In methanol-induced cells of each *per* mutant, substantial activity for both alcohol oxidase (AOX) and catalase (CAT) was present

A. Peroxisomal Enzyme Activities

	wt	1	3	5	7	8	9	10	11	12	13	14
CAT	100	83	103	163	79	121	190	81	26	133	43	211
AOX	100	59	134	122	66	34	72	25	24	100	42	218

B. Sedimentation Patterns of Peroxisomal Enzymes

Fig. 3A,B Activity and location of peroxisomal enzymes in methanol-induced *per* mutant cells. **A** percentage of AOX and CAT activities relative to that in wild-type cells. **B** percentage of AOX, CAT and fumarase activities in the post-25000 g organelle pellet after differential centrifugation of homogenized protoplasts

(Fig. 3A). Homogenized protoplasts prepared from these cultures were then subjected to differential centrifugation and the sedimentation pattern of AOX and CAT determined (Fig. 3B). In wild-type preparations, approximately 60% of AOX and 40% of CAT activity was present in the organelle pellet. A significant amount of activity was also present in the supernatant due to organelle breakage during the procedure. However, with each of the *per* mutant preparations, most AOX and CAT activity was found in the supernatant, indicating that the enzymes were located in the cytosol. These results were not a consequence of unequal handling of *per* cells during preparation since fumarase, a mitochondrial marker enzyme, remained primarily in the pellet.

The cytosolic location of AOX and CAT was confirmed by immunocytochemical experiments. In methanol-induced *Per*⁻ cells, AOX typically formed a large cytosolic crystalloid (Fig. 4A) that was readily observed by light microscopy (inset to Fig. 4B). In contrast, CAT appeared to be randomly dispersed throughout the cytosol in each mutant (data not shown). Dihydroxyacetone synthase (DHAS), the third peroxisomal methanol-pathway enzyme, was also present and located primarily within the crystalloid in each mutant (Fig. 4B).

Fig. 4A, B Micrographs of a methanol-induced Per^- strain (C111, *per7*). **A** EM detail of an ultrathin cryosection of the strain demonstrating the cytosolic AOX crystalloid; **A inset**, characteristic immunofluorescent pattern observed after incubation of the strain with antibodies specific for total PMPs and FITC. **B** accumulation of DHAS protein in AOX crystalloids revealed through immunocytochemical staining using specific antibodies against DHAS and protein A gold; **B inset**, phase-contrast light microscopy of AOX crystalloids in induced Per^- cells. Bar, 1 μ m. Light micrographs are at 3000 x

Identification and analysis of dominant-negative mutants

For two Per^- strains, C4 and C35, we were unable to introduce an auxotrophic marker by mating and, therefore, could not perform complementation testing initially. Their apparent failure to mate may have been the result of the presence of secondary mutations affecting this process or of dominant-negative (PER^D) alleles which would prevent the growth and selection of diploids on minimal methanol medium (a critical requirement of mating homothallic yeasts such as *H. polymorpha* is the ability to selectively grow crossed diploid lines in the presence of self-mated diploids and non-mated parental cells.) To further investigate these mutants, spontaneous uracil-requiring (Ura^-) derivatives of C4 and C35 were isolated by selection for resistance to 5-fluoro-orotic acid (Boeke et al. 1987). The Ura^- derivatives were then mated with auxotrophically marked strains and observed to readily form prototrophic diploids, a result that suggested the presence of a PER^D allele in C4 and C35. The dominant-negative nature of the alleles was confirmed by the observation that C4/wt and C35/wt diploid strains were Mut^- . Further testing revealed that C35/wt diploids were actually cold sensitive (Mut^- at 25 and 30 $^{\circ}$ C but Mut^+ at 37 and 43 $^{\circ}$ C), whereas C4/wt strains were Mut^- at all temperatures tested (25 to 43 $^{\circ}$ C).

Peroxisome morphology in methanol-induced cultures of diploid C4/wt and C35/wt strains was examined by EM. Control diploid strains, constructed from crosses between strains with recessive *per* alleles and either wild type or complementing *per* allele pairs, contained normal peroxisomes (Fig. 5 A). In contrast, C4/wt diploids contained only a few small peroxisomes along with a large cytosolic AOX crystalloid (Fig. 5 B), a phenotype similar to that seen in haploid Pim^- mutants of *H. polymorpha* (Waterham et al. 1992). C35/wt diploids which were cold sensitive for methanol growth were also cold sensitive with respect to peroxisome development with normal-appearing organelles at 43 $^{\circ}$ C (Fig. 5 C). At 25 $^{\circ}$ C, C35/wt diploids contained few large peroxisomes. Furthermore, the large mature organelles frequently displayed distinctive discontinuities in their membranes (Fig. 5 D). It is difficult to understand how such membrane holes could exist in vivo. However, since they were not observed in wild-type control samples, they could not be entirely due to preparation artifacts. Perhaps the peroxisomal membrane is weak at one or more points and the stress of sample preparation then disrupts the membrane at these locations.

The PER^D alleles were assigned to *PER* gene groups by first crossing Ura^- derivatives of C4 and C35 to Leu^- -marked recessive *per* representatives of each group and selecting for diploids on minimal glucose medium. As expected, all of the resulting diploids were Mut^- . Diploid strains derived from each cross were then sporulated and approximately 1000 spore products from each cross were examined for Mut phenotype. Approximately 25% of the spore products were Mut^+ from all but two crosses. The two exceptions were C4 crossed with the *PER5* representative, and C35 crossed with the *PER7* representative, which both yielded no Mut^+ spores. The absence of Mut^+ spore products demonstrated tight linkage between alleles and indicated that the mutations

Fig. 5A–D Electron micrographs of selected methanol-induced *H. polymorpha* diploid strains. **A** Normal peroxisomes in a diploid constructed from two recessive *Per*[−] alleles *per2* and *per7*. **B** C4/wt (*PER5^D/PER5*) diploid cell showing only a few small peroxisomes in conjunction with a single large cytosolic AOX crystalloid (Lowicryl, anti-AOX antibodies, protein A/gold). **C** C35/wt (*PER7^D/PER7*) diploid cell cultured in methanol at 43°C showing normal peroxisome proliferation and morphology. **D** The same strain in C cultured in methanol at 25°C. Note the interruption in the membrane of the mature peroxisome (arrow). Bar, 1 μ m

in C4 and C35 were *PER^D* alleles of *PER5* and *PER7*, respectively.

Discussion

In previous reports, we established that functional peroxisomes are essential to support growth of *H. polymorpha* on methanol as a sole carbon and energy source, and that mutants with defects in the organelle

(*per* mutants) can be found among collections of *Mut*[−] mutants of this yeast (Cregg et al. 1990; van der Klei et al. 1991 a). We have exploited this discovery by isolating and characterizing 60 *per* mutants. The mutants can be differentiated into three phenotypic classes based on peroxisome morphology in methanol-induced mutant cells. Two mutants contain peroxisomes in which the normally ordered structure of the crystalline matrix, primarily composed of AOX, is disorganized (Titorenko et al. 1993). This phenotype is named *Pss*[−] for peroxisomes with abnormal matrix substructure. The two *Pss*[−] mutant alleles are in *PER3* and *PER6*. Another 20 *per* strains contain only very small peroxisomes (Waterham et al. 1992). In methanol-induced cells of these mutants, peroxisomal matrix enzymes are present and active. However, only a fraction is imported into the small organelles. This phenotype is named *Pim*[−] for peroxisome import defective. We previously described *Pim*[−] alleles in five genes, *PER1* through *PER5*.

In the present report, we describe the identification and characterization of the third phenotypic class of *per* mutants which we call Per^- . Methanol-induced cells of each Per^- mutant were carefully searched by EM for peroxisome-like structures but none could be found. Thus, either Per^- mutants do not contain peroxisomes or they are unrecognizable. In Per^- mutants, the peroxisomal enzymes AOX, CAT and DHAS are present but mislocalized to the cytosol. As also seen in Pim^- mutants, AOX forms into a single active non-membrane-bounded crystalloid structure (van der Klei et al. 1991b). One Per^- mutant, C111 (a *PER7* representative), has been examined for the fate of peroxisomal membrane proteins (PMPs) (Sulter et al. 1993b). In this mutant, PMPs exist as part of a cytosolic proteinaceous phospholipid aggregate in each cell. The aggregate, which is thought to be a possible peroxisomal remnant, can be observed by immunofluorescence microscopy as a punctate structure within C111 cells (inset to Fig. 4A). Similar punctate structures are observed in each Per^- mutant, suggesting that PMP-containing aggregates are a common feature of this class of *per* mutant.

Two of the Per^- mutants harbor dominant-negative mutant (*PER^D*) alleles. Diploid strains constructed between either of these mutants and wild-type *H. polymorpha* are Mut^- . One, C35 (*PER7^D*), forms heterozygous diploids that display a cold-sensitive Mut^- phenotype. The other, C4 (*PER5^D*), forms diploids that are Mut^- at all temperatures. Consistent with their Mut^- phenotype, peroxisome morphology in methanol-induced diploid cells is also abnormal, although in both cases the morphology is different from that of the haploid parents. Whereas both C4 and C35 are Per^- , the *PER5^D/wt* diploid contains small peroxisomes along with a large cytosolic AOX crystalloid, while the *PER7^D/wt* diploid contains peroxisomes with membrane disruptions at 25 °C and normal organelles at 37 °C. Both *PER^D* alleles are in genes for which numerous recessive *per* alleles also exist and their isolation suggests that the affected *PER* products take part in important protein-protein interactions either with themselves as homomultimers or with other peroxisomal proteins. These are the first dominant mutations affecting peroxisome biogenesis to be reported and the first dominant negative mutants to be described in a methylotrophic yeast species. Furthermore, only a few reports of dominant negative mutants affecting organelle biogenesis exist in the literature (Robinson et al. 1988; Vater et al. 1992).

It is tempting to speculate on the nature of the gene products whose defects could lead to a Per^- phenotype, i.e., the complete absence of the organelles. However, few clues are available for meaningful conjecture. The mutants could be defective in the peroxisome fission process, in the segregation of the organelles to daughter cells, in a protein recognition/import system,

or in regulatory factors involved in expression of other products required for peroxisome biogenesis. The Per^- mutants are most likely not defective in factors required for catabolite de-repression such as the *SNF* products, as are several of the *S. cerevisiae* peroxisome-deficient (*pas*) mutants (Simon et al. 1992; van der Leij et al. 1992), since all our *per* mutants grow normally on other de-repression-requiring carbon sources including ethanol and glycerol, and induce relatively normal levels of methanol-pathway enzymes.

We would also like to determine the functional significance, if any, of the Per^- , Pim^- and Pss^- phenotypic classes. Evidence, suggests that, in certain cases, Per^- is the phenotype of a totally defective *PER* gene while Pim^- is the result of a partial or "leaky" mutation. First, several instances have been found in which different mutant alleles in the same *PER* gene result in strains with different peroxisome phenotypes (Titorenko et al. 1992; this report). As examples, mutant alleles of *PER1* and *PER5* can be either Per^- or Pim^- while *PER3* mutant alleles are either Pim^- or Pss^- . Second, two *per* mutants (*per3* and *per13*) which were initially identified as Per^- became Pim^- after backcrossing. Third, a temperature-sensitive *per* mutant that is Per^- at non-permissive temperature displays a Pim^- phenotype when cultured at an intermediate temperature (Waterham et al. 1993). However, it is also possible that some *PER* genes are involved in more than one function in peroxisome biogenesis and yield mutant alleles that are specifically defective in each function. A definitive answer for each *PER* gene must await the construction of null alleles from the cloned genes.

Among yeast species, *H. polymorpha* has several unique advantages as a model system for molecular genetic studies on peroxisomes (Veenhuis 1992). Due to the requirement of peroxisomes for growth on methanol and the large size of the organelles in methanol-induced cells, *per* mutants are relatively easy to isolate. The task of identifying *per* mutants is made even easier by the observation that methanol-induced *per* mutant cells (Per^- and Pim^- classes) uniquely contain a large AOX crystalloid which is readily visible under the light microscope (van der Klei et al. 1991b). Thus, *per* mutants can be efficiently identified within Mut^- collections of *H. polymorpha* by light microscopy, while EM observation can be reserved for confirmation of a peroxisome-deficient diagnosis. Another unusual but useful characteristic of *H. polymorpha* is its relative thermo-tolerance. The yeast grows well at temperatures of up to 45 °C, which is particularly advantageous for isolating temperature-sensitive *per* mutants. With temperature-sensitive *per* alleles it is possible to observe the biogenesis of new peroxisomes, by shifting cultures from non-permissive to permissive temperature, or to watch the effects of the withdrawal of a required peroxisomal product by shifting from permissive to nonpermissive temperature (Sulter et al. 1993b; Waterham et al. 1993).

To-date we have identified mutant alleles in 14 different *PER* genes. The existence of this large collection of *H. polymorpha* per mutants, along with efficient transformation vectors and host strains for this yeast, have allowed us to take the next major step in our studies, the cloning of *H. polymorpha* *PER* genes by functional complementation. Several *PER* genes have been isolated to-date (Waterham et al. 1994; unpublished data). The predicted amino-acid sequences of *PER* products, along with detailed biochemical information on the effect of their malfunction on the cell, should provide useful insights into the function of specific *PER* products and their roles in peroxisome biogenesis.

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