Identification of intragenic mutations in the *Hansenula polymorpha* PEX6 gene that affect peroxisome biogenesis and methylotrophic growth

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Abstract

Two interacting AAA ATPases, Pex1p and Pex6p, are indispensable for peroxisome biogenesis in different organisms. Mutations affecting corresponding genes are the most common cause of the peroxisome biogenesis disorders in humans. By UV mutagenesis of the *Hansenula polymorpha* pex6 mutant, deficient in peroxisome biogenesis, we isolated a conditional cold-sensitive strain with restored ability to grow in methanol medium at 37°C but not at 28°C. Sequencing of the pex6 allele revealed a point mutation in the first AAA module of the PEX6 gene that leads to substitution of a conserved amino acid residue (G737E). An additional intragenic mutation identified in the cold-sensitive pex6 allele leads to a conserved amino acid substitution in the second AAA domain (R1000G). Electron microscopic analysis revealed restored peroxisomes in methanol-induced cold-sensitive pex6 cells at both permissive and restrictive temperatures. If separated, the secondary mutation did not affect methylotrophic growth. Our data suggest that *H. polymorpha* Pex6p may have a complex function in peroxisome biogenesis in which identified amino acid residues are involved.

Keywords: Peroxisome biogenesis; PEX6; Methylotrophic yeast; *Hansenula polymorpha*

1. Introduction

Accumulated data suggest that mechanisms of peroxisome biogenesis are well conserved among different eukaryotes (reviewed in [1,2]). Yeasts have proved to be a convenient model system to study molecular mechanisms of peroxisome biogenesis. In yeasts, peroxisomes are dispensable for growth unless particular carbon sources, i.e. oleate, methanol, are utilized. Therefore, mutants deficient in peroxisome biogenesis could be efficiently isolated and studied in different yeast species [1–4]. Methylotrophic yeasts have a particular advantage in studying mechanisms of peroxisome biogenesis because of the massive peroxisome proliferation they exhibit upon induction by methanol [5].

Among genes involved in peroxisome biogenesis (PEX genes, their products designated peroxins) originally discovered in yeasts are *PEX1* and *PEX6* that encode ATPases belonging to an AAA family [1,6,7]. It was demonstrated that mutations in corresponding human homologues are the most common cause of inherited peroxisome biogenesis disorders (PBD) [8]. The function of the peroxins involved has not been completely elucidated. In yeasts and higher eukaryotes Pex1p and Pex6p physically and functionally interact in vivo, and this interaction is ATP-dependent [8–10]. Based on the model of the yeast *Yarrowia lipolytica*, it was proposed that the Pex1p/Pex6p complex participates in the fusion events of preperoxisomal vesicles [11]. Other data suggest that this complex is required for terminal steps of matrix protein import [12].

In the methylotrophic yeast *Hansenula polymorpha*, de-
iciency in the PEX6 gene impairs peroxisome matrix protein import: upon methanol induction membranous peroxisome 'ghosts' are present in the cells and peroxisomal enzymes, such as alcohol oxidase and catalase, are mislocalized to the cytosol [9]. As a result, pex6 mutants are unable to utilize methanol as carbon and energy source. In this report, we describe a conditional cold-sensitive (cs) pex6 mutant strain isolated upon introduction of an additional intragenic mutation in the PEX6 gene of a pex6 mutant, and identification of the corresponding mutations. Preliminary data on its isolation have been published [13].

2. Materials and methods

2.1. Yeast strains and media

The H. polymorpha strains used were: the wild-type strains CBS4732 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands),NCYC495 leu1-1 (National Collection of Yeast Cultures, Food Research Institute, Norwich, UK); pex6 leu1-1 mutant, originally designated per5-C79 [14], and Δpex6 strain [9].

Yeasts were grown at 28°C or 37°C in YPD medium (1% yeast extract, 1% peptone and 1% glucose) or selective mineral media (MM) with 0.05% yeast extract [15], supplemented with 1% glucose, 0.5% methanol, 1% ethanol or 0.5% glycerol and 0.5% ammonium sulfate as a nitrogen source. Leucine (40 mg l⁻¹) was added to all mineral media. Yeast cell density was determined by absorbance at 590 nm. For solid media agar (2%) was added. Sporulation/mating media and techniques for H. polymorpha were essentially as described [16].

2.2. Molecular techniques

Procedures and conditions used for isolation of plasmid DNA, digestion with restriction enzymes, isolation of DNA fragments, and agarose gel electrophoresis were carried out according to [17]. Restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA) and used as indicated by the supplier. The Escherichia coli strain DH5α was used for plasmid propagation.

Electrotransformation of H. polymorpha was performed as described [18]. To isolate genomic DNA fragments with mutated PEX6 genes, total DNAs were isolated from pex6 and cs pex6 mutants as described [19]. They served as templates in polymerase chain reactions (PCR) with the primer PEX6-F: 5'-GCTGTCGTCAACAATAATG-3', specific for the PEX6 gene promoter region, and the primer PEX6-R: 5'-ATTTACCGGAAGTCGCTTG-3', specific for the sequence downstream of the PEX6 gene. PCR was carried out with Taq-DNA Polymerase (Promega, Madison, WI, USA) according to the supplier's instructions. Nucleotide sequencing of the isolated PCR products was carried out as described [9]. To exclude potential PCR amplification mistakes, two independently amplified fragments for each strain were sequenced in both directions.

Sequence alignments were performed using the MultAlign software [20]. ORF Finder, a graphical analysis tool of the National Center for Biotechnology Information (Bethesda, MD, USA) was used for analysis of DNA sequences. Protein sequence homology search was done using the WWW BLAST server.

2.3. Conditional mutant isolation

For mutant isolation, cells of the initial pex6 strain were grown in liquid YPD, washed twice with distilled water, spread on the MM plates with 0.5% methanol (5×10⁷ cells per plate), and UV-mutagenized for 60 s. Plates were incubated for 4-5 days at 28°C or 37°C and growing colonies were then replica-plated on the same medium and incubated at different temperatures to identify conditional mutants.

2.4. Electron microscopic methods

Cells were fixed and prepared for electron microscopy as in [15].

Fig. 1. Growth kinetics of H. polymorpha wild-type strain, pex6 and cs pex6 mutants in methanol medium at (A) 37°C and (B) 28°C.
3. Results

3.1. Isolation of the cs pex6 mutant

We initiated experiments aimed at isolating UV-induced, conditional (ts or cs) revertants from an H. polymorpha pex6 (per5-C79) mutant as an initial strain. The pex6 cells were mutagenized, and colonies with restored ability to grow on MM methanol plates were selected. It appeared that stable revertants can be isolated from per5-C79 at a high frequency of $1 \times 10^{-6}$. Out of several hundred isolated UV-induced revertants, one conditional (cs) clone with restored growth on methanol at 37°C (permissive temperature) and very slow growth at 28°C (restrictive temperature) was identified (Fig. 1). This cs pex6 mutant exhibited the cold-sensitive phenotype only in methanol medium, but not in glucose, ethanol or glycerol media (not shown). Therefore, presumably a secondary mutation causative for the conditional phenotype interfered only with methylotrophic growth.

3.2. Ultrastructural characterization of the cs pex6 strain

To elucidate whether the restored ability to utilize methanol in the cs pex6 mutant is accompanied by restored peroxisome biogenesis, electron microscopic analysis was carried out. It revealed morphologically detectable peroxisomes at both permissive and restrictive temperatures upon methanol induction (Fig. 2). Peroxisomes with the wild-type morphology were induced in cs pex6 cells at the permissive temperature, 37°C (Fig. 2F). No significant difference was observed for the mutant relative to the wild-type strain with regard to peroxisome size, peroxisome number per cell, or activity of the major peroxisome ma-

Fig. 2. Morphology of H. polymorpha wild-type strain (A,B), pex6 (C,D) and derivative cs pex6 (E,F) mutants incubated in methanol MM for 12 h at 28°C (A,C,E) and 37°C (B,D,F). M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 1 μm.

Fig. 3. Schematic representation of HpPex6p structure with sites of identified mutated residues. A: Two predicted nucleotide binding domains or AAA modules (in gray) are denoted as AAA1 and AAA2 with indicated numbers of flanking amino acid residues. Motifs characteristic of the AAA family of ATPases Walker A and B (WA, WB), and minimal AAA or ‘second region of homology’ (SRH) are shown. Mutated nucleotides and corresponding amino acid residues are shown in bold.

B: Fragments of alignment of yeast and human Pex6 and Pex1 proteins comprising mutated residues (in bold). Proteins aligned were from H. polymorpha (Hp), Pichia pastoris (Pp), Saccharomyces cerevisiae (Sc), Yarrowia lipolytica (Yl), and Homo sapiens (Hs).
trix enzyme alcohol oxidase upon methanol induction at 37°C.

At 28°C, the restrictive temperature for methylotrophic growth, morphologically altered enlarged peroxisomes were observed in most of the thin sections (Fig. 2E). Thus, a secondary mutation in cs \textit{pex6} restored peroxisome formation at the restrictive temperature, but not organelar function necessary for methylotrophic growth.

3.3. Genetic analysis

The cs \textit{pex6} mutant was crossed with the wild-type strain, and the resulting spore progeny of a diploid hybrid strain was analyzed for Pex⁻ phenotype. Out of several thousand individual colonies only one was identified as unable to grow on methanol at 28 and 37°C. In all other cases colonies exhibited wild-type or cs-conditional parental phenotypes. This suggested cosegregation of an initial and a secondary mutation. Therefore, the corresponding cs mutation was most probably tightly linked with, or resided in, the \textit{PEX6} locus.

3.4. Identification of the mutations

Genomic DNA fragments of 3600 bp comprising the \textit{PEX6} gene were isolated by PCR from initial \textit{pex6} and derivative cs \textit{pex6} mutants as described in Section 2. Their sequencing revealed a missense mutation in the \textit{PEX6} gene of \textit{pex6} allele (G2210A) that leads to an amino acid substitution G737E in the deduced Pex6p. In addition to the above mutation, the second intragenic mutation for the cs \textit{pex6} allele was identified as an A2998G transition, leading to an amino acid substitution R1000G.

The glycine G737 residue mutated in the initial \textit{pex6} strain is conserved in yeasts and human Pex6p homologues and resides in the C-terminal part of the first AAA module of Pex6p (Fig. 3). Moreover, it is also conserved in the first AAA module of the \textit{H. polymorpha} and human Pex1p, an interacting partner of Pex6p (Fig. 3B). This suggests the functional importance of the glycine residue at this position in Pex6p and Pex1p in lower and higher eukaryotes.

The arginine R1000 residue, mutated in cs \textit{pex6}, resides in the second AAA module of Pex6p and is conserved only among yeast and human Pex6p homologues, but not in yeast and human Pex1p (Fig. 3). This may imply functional importance of this residue specifically for Pex6p homologues.

3.5. Complementational analysis of cs \textit{pex6} mutant

Unexpectedly, both \textit{pex6} and cs \textit{pex6} strains could not be fully rescued to the wild-type phenotype when transformed with a replicative plasmid pYT3-PEX6, isolated from a \textit{H. polymorpha} genomic library and harboring the wild-type copy of the \textit{PEX6} gene [9] (Fig. 4). Similar growth deficiency on methanol was also exhibited by the hybrid diploid strains resulting from crosses of \textit{pex6} or cs \textit{pex6} mutants with the wild-type strain. The diploid carrying the mutant \textit{pex6} and cs \textit{pex6} alleles of the gene exhibited the cold-sensitive phenotype (Fig. 4). However, the plasmid pYT3-PEX6 fully complemented the mutant with deleted \textit{PEX6} gene (Fig. 4). The results suggest that G737E substitution leads to a partial dominant-negative phenotype, more pronounced at 28°C.

To elucidate the phenotype, caused independently by
the R1000G substitution, we transformed the cs pex6 mutant with two di¡erent linear fragments of the wild-type PEX6 gene isolated from the plasmid pYT3-PEX6 by restriction enzyme digestion. The 2080-bp-long AatII fragment comprised a portion of the PEX6 open reading frame with the site of the G2210A missense mutation in pex6 (Fig. 5). The second 2199-bp-long ClaI fragment harbored the C-terminal portion of the PEX6 gene with the sites of both mutations identified in cs pex6 (G2210A, A2998G). When transformed in pex6 and cs pex6 mutants by electroporation, both fragments complemented growth de¢ciency on methanol in these strains at 28°C, as well as at 37°C. All isolated transformants were stable with respect to methlyotrophic growth, suggesting that homologous recombination occurred in the PEX6 locus. Several corresponding transformants of the cs pex6 mutant were analyzed for kinetics of growth in methanol medium (Fig. 6, with data for the two representative transformants provided). It was demonstrated that strains transformed with the ClaI fragment, able to complement both missense mutations, have wild-type kinetics of growth at 28°C and 37°C. AatII fragment transformants exhibited some retardation in growth at 28°C. These results suggest that complementation of the G737E substitution in cs pex6 restores its ability to utilize methanol at the restrictive temperature. Secondly, R1000G substitution alone does not affect methylotrophic growth and peroxisome biogenesis at 37°C, and does so only to a minor extent at 28°C.

4. Discussion

In this report we describe the identi¢cation of the missense mutation in the H. polymorpha PEX6 gene that leads to de¢ciency in peroxisome biogenesis and methylotrophic growth and a dominant-negative phenotype. The corresponding mutation causes an amino acid substitution in the first AAA module of the Pex6p (G737E). We also demonstrated that this mutation can be rescued to a conditional cs phenotype by introducing a secondary intragenic mutation in the second AAA module of Pex6p (R1000G). Both altered amino acid residues are conserved among yeast and human Pex6p homologues.

These residues are situated at C-termini of AAA modules, outside the known functionally important regions of core Walker A, Walker B, and C-terminal Sensor 1 and Sensor 2 motifs [21]. To our knowledge, mutations in the corresponding segments have not been identi¢ed or constructed for other studied AAA proteins, including human Pex1p and Pex6p homologues. C-termini in AAA modules have an 8-helical composition and have been proposed to transduce changes in the nucleotide state to speci¢c conformational changes and domain movements [22]. Interestingly, while G737E mutation abolishes Pex6p function, R1000G substitution has no signi¢cant effect when present alone. Both identi¢ed amino acid substitutions in HpPex6p involve a change in charge of a residue at the corresponding position (G/E, R/G). From studies of three-dimensional structures of crystallized AAA proteins and mutational analysis it is known that the two AAA modules are independent domains [23,24]. It is tempting to speculate that a complementary effect of the R1000G substitution on the G737E background is indicative of cross-
talk between the two Pex6p AAA modules, of either intramolecular or intermolecular character. It is also not excluded that these mutations affect Pex6p/Pex1p interaction, indispensable for peroxisome biogenesis.

The dominant character of the G737E mutation suggests that the presence of such mutated Pex6p in the cell somehow disrupts peroxisome functioning even when the wild-type copy of the protein is introduced. This pex6 mutant will be subjected to further studies, which may provide new clues on molecular aspects of Pex6p functioning in peroxisomal import of matrix proteins.

The deficiency in methylotrophic growth in the cs pex6 strain at the restrictive temperature is not caused by a defect in peroxisome formation, as demonstrated by the electron microscopic experiments. Restored morphologically detectable peroxisomes appear to be deficient in one of their functions at the restrictive temperature, implying a complex role of Pex6p in organelle biogenesis. Further studies will be aimed at elucidating the molecular background of the conditional phenotype in the double mutant. This strain is more sensitive to elevated concentrations of methanol than the wild-type strain, and its growth on methanol at 28°C cannot be rescued by over-expression of HpPex1 or HpPex5 genes (data not shown). These elaborations suggest that deficiency in methylotrophic growth in cs pex6 may result from the mislocalization of peroxisomal catalase [4].

Cs pex6 mutant cells exhibited the wild-type rate of inactivation of peroxisomal alcohol oxidase when shifted from methanol to glucose medium, at both restrictive and permissive temperatures [13]. Therefore, Pex6p is not involved in autophagic peroxisome degradation, which is permissive at thermostable temperatures [13]. Hence, Pex6p is not involved in the terminal steps of peroxisomal matrix protein import.

This suggests that the presence of such mutated Pex6p in the cell somehow disrupts peroxisome functioning even when the wild-type copy of the protein is introduced. This pex6 mutant will be subjected to further studies, which may provide new clues on molecular aspects of Pex6p functioning in peroxisomal import of matrix proteins.

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Studies of the missense mutations in the PEX6 gene may lead to a better understanding of its function in peroxisome biogenesis in yeasts, as well as in humans. Mutations in the human PEX6 homologue (HsPex6) are the second most common cause, after HsPex1, of PBD (groups CG4, CG6) [8,26]. It has been demonstrated that nonsense mutations, deletions and those mutations affecting splicing severely affect HsPex6p function and lead to the lethal Zellweger syndrome [27,28]. Missense mutations in HsPex6, as well as in HsPex1, cause milder forms of PBD such as neonatal adrenoleukodystrophy and infantile Refsum disease [27,29,30]. In many cases, such mutations exhibit a temperature-sensitive phenotype, and peroxisome biogenesis can be restored in patients' fibroblasts at lowered temperatures [28,30,31]. In this respect, it should be noted that most of the missense mutations in HsPex6 and HsPex1 actually disrupt interaction of their protein products [8,32]. Studies have been initiated to develop pharmacological approaches for treating patients with such milder forms of PBD [33]. Therefore, yeast mutants, including those with introduced known human mutations, can serve as a convenient model system for studying molecular mechanisms of functioning of Pex6p and other PEX products in peroxisome biogenesis.

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