The *Hansenula polymorpha* PER1 Gene Is Essential for Peroxisome Biogenesis and Encodes a Peroxisomal Matrix Protein with Both Carboxy- and Amino-terminal Targeting Signals

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Abstract. We describe the cloning of the *Hansenula polymorpha* PER1 gene and the characterization of the gene and its product, PERlp. The gene was cloned by functional complementation of a per1 mutant of *H. polymorpha*, which was impaired in the import of peroxisomal matrix proteins (Pim- phenotype). The DNA sequence of PER1 predicts that PERlp is a polypeptide of 650 amino acids with no significant sequence similarity to other known proteins. PER1 expression was low but significant in wild-type *H. polymorpha* growing on glucose and increased during growth on any one of a number of substrates which induce peroxisome proliferation. PERlp contains both a carboxy- (PTS1) and an amino-terminal (PTS2) peroxisomal targeting signal which both were demonstrated to be capable of directing bacterial β-lactamase to the organelle. In wild-type *H. polymorpha* PERlp is a protein of low abundance which was demonstrated to be localized in the peroxisomal matrix. Our results suggest that the import of PERlp into peroxisomes is a prerequisite for the import of additional matrix proteins and we suggest a regulatory function of PERlp on peroxisomal protein import.

Eukaryotic cells are characterized by the compartmentalization of various metabolic functions into separate subcellular organelles. Each organelle contains a characteristic set of proteins to accomplish specific metabolic functions essential for the cell. Microbodies (peroxisomes, glyoxysomes) represent the most recently discovered class of organelles, which are ubiquitous in higher and lower eukaryotic organisms (Borst, 1989; van den Bosch et al., 1992). They are involved in a variety of metabolic functions (Lazarow and Kindl, 1982; Veenhuis and Harder, 1991; van den Bosch et al., 1992) and in many cases their presence appears to be essential for the cell's viability. Consequently, the organelles have been intensively studied and in recent years the knowledge on the molecular mechanisms of microbody biogenesis and function is rapidly expanding.

It is now generally accepted that upon their induction microbodies develop by multiplication of preexisting organelles. Microbody proteins are encoded by nuclear genes, synthesized on free cytosolic polysomes to their mature size, and posttranslationally translocated into their target organelles (Lazarow and Fujiki, 1985; Borst, 1989). At present two different peroxisomal-targeting signals (PTS) have been identified which are capable of directing matrix proteins to the organelles. The first one (designated PTS1) resides at the extreme carboxy terminus of the protein and includes the tripeptide motif SKL or degenerate forms of it. The PTS1 motif has been shown to serve as a general microbody targeting sequence in animals, plants, and yeasts (Gould et al., 1989, 1990; Swinkels et al., 1992). However, in particular in yeast, additional variations of the PTS1 motif were found indicating that more extensive degenerations of the original PTS1 are functional in these organisms (de Hoop and AB, 1992). Examples of these include the carboxy-terminal tripeptides AKI (Aitchison et al., 1991), SKI (Diodon and Roggenkamp, 1992), SKF (Kragler et al., 1993), ARE and NKL (Hansen et al., 1992) which act as a PTS1 in different yeast species. Subsequently, a second peroxisom...
mal-targeting signal was identified, located at the amino terminus of rat peroxisomal thiolase (Osumi et al., 1991; Swinkels et al., 1991). Sequence comparison with other peroxisomal matrix proteins, lacking a distinct PTS1, revealed a consensus sequence RLxQ/I-IL which is observed in the amino terminus of various thiolases, watermelon malate dehydrogenase, *Hansenula polymorpha* amine oxidase, and *Trypanosoma brucei* aldolase (de Hoop and AB, 1992). Recently, evidence was obtained that this conserved sequence (designated PTS2) indeed serves as a peroxisomal-targeting signal in watermelon malate dehydrogenase (Gietl et al., 1994) and *H. polymorpha* amine oxidase (Faber, K. N., I. Keizer-Gunnihh, C. Pluim, W. Harder, G. AB, and M. Veenhuis, manuscript submitted for publication).

A further increase in our knowledge of microbody biogenesis is to be expected from the analysis of the various peroxisome-deficient yeast mutants as they have been isolated from *Saccharomyces cerevisiae* (Erdman et al., 1989; van der Leij et al., 1992; Elgersma et al., 1993; Zhang et al., 1993), *H. polymorpha* (Cregg et al., 1991; Veenhuis, 1992; Waterham et al., 1992b), *P. pastoris* (Gould et al., 1991; Liu et al., 1992), and *Yarrowia lipolytica* (Nuttley et al., 1993). A major advantage of these mutants is that, in contrast to other cell organelles, the complete absence of peroxisomes does not affect the viability of the cells. Therefore, peroxisome-deficient yeast mutants provide excellent models to dissect the molecular mechanisms of peroxisome biogenesis. At present several genes, essential for peroxisome biogenesis, have been cloned and characterized by functional complementation of the corresponding mutants (Erdmann et al., 1991; Höhfeld et al., 1991, Wiebel and Kunau, 1992; McCollum et al., 1993; Spong et al., 1993).

Recently, we have cloned several *H. polymorpha* PER genes by functional complementation of various peroxisome-deficient mutants of this organism. Here, we report the molecular cloning and sequencing of the PERI gene and the characterization of its product.

**Materials and Methods**

**Strains and Plasmids**

*H. polymorpha* and *Escherichia coli* strains and plasmids used in this study are listed in Table I.

**Cultivation Media and Growth Conditions**

*E. coli* strains used for molecular manipulations were grown as described (Sambrook et al., 1989).

*H. polymorpha* strains were grown at 37°C in selective minimal YND or YNM media (0.7% [wt/vol] DIFCO [Detroit, MI] Yeast Nitrogen Base without amino acids supplemented with 1% [wt/vol] dextrose [glucose] or 0.5% [wt/vol] methanol, respectively), in YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 1% [wt/vol] dextrose), or in mineral medium (Veenhuis et al., 1989) supplemented with 0.5 or 1% (wt/vol) carbon source and 0.25% (wt/vol) nitrogen source. In addition, cells were grown in continuous culture at 37°C in mineral medium (van Dijken et al., 1976) using a mixture of 0.25% (wt/vol) glucose and 0.2% (vol/vol) methanol as carbon/energy source. Amino acids and uracil were added to a final concentration of 40 μg/ml.

For growth on agar plates all media were supplemented with 1.5% (wt/vol) granulated agar.

**Isolation and Characterization of Perl Mutants**

The isolation, back-crossing, complementation analysis, gene mapping, and phenotype characterization of various *H. polymorpha* per mutants, including perI mutants, are detailed in previous papers (Veenhuis, 1992; Waterham et al., 1992b; Titorenko et al., 1993).

**Construction of a H. polymorpha genomic DNA Library**

Chromosomal DNA from YPD-grown wild-type (WT) *H. polymorpha* harvested at O.D600-1.5, was isolated essentially as described by Sherman et al. (1986). A sized fraction of genomic DNA fragments ranging from 5 to 10 kb was obtained by partial digestion of chromosomal DNA with *Sac3A*, followed by fractionation on a sucrose gradient (seven layers of 1.7 ml sucrose in 10 Mm Tris/1 Mm EDTA [pH 7.5], increasing in steps of 3% from 10 to 40% [wt/vol], using a Beckman Instrs. (Carlsbad, CA) SW41 rotor (20 h, 25,000 rpm at 20°C). The sized DNA fragments were ligated in the phagemid system of Promega. For sequencing, segments of the 3-kb complementing DNA fragment were subcloned in PBSII KS + using restriction sites present in phi8R2-vector) in the unique SmaI-site of the phungemid pBluescript KS + plasmid. After transformation, 2×10^6 clones with an average insert size of 6 kb were obtained. Using the formula of Clarke and Carbon (1976), the chance for a random DNA sequence to be present in the obtained DNA library amounts to 99% (the total *H. polymorpha* genomic DNA size was estimated to be 2×10^9 bp).

**Cloning of the PERI Gene**

To isolate the PERI gene, a three-times backcrossed strain of mutant perI-124/D (Waterham et al., 1992b) was transformed with the constructed genomic DNA library (Faber et al., 1992). After 4-5 d growth on YN agar plates, leucine prototrophic transformants were replica-plated onto YNM-agar plates and screened for the ability to utilize methanol (growth generally within 3-5 d). Plasmids were recovered by electrotransformation of *E. coli* MC1061 with plasmid DNA isolated from complemented *H. polymorpha* strains (Faber et al., 1992). As a control the original perI-124/D strain was retransformed with the isolated plasmids, again followed by plasmid recovery in *E. coli*. To facilitate restriction analysis and construction of subclones, the complementing genomic DNA fragment was subcloned as a T4 phosphatase-treated Nhel-SphI fragment (both sites exclusively present in phi8R2-vector) in the unique SmaI-site of the phagemid pBluescript II KS + (pBSII KS +) and isolated in two different orientations.

**DNA Sequencing and Analysis**

Sequencing was performed in two directions by double-stranded sequencing according to the dideoxy method (Sanger et al., 1977) using the DeazaT sequencing kit of Pharmacia LKB Biotechnologie and the TAQ Track Sequencing System of Promega. For sequencing, segments of the 3-kb complementing DNA fragment were subcloned in PBSII KS + using restriction sites present on the fragment. The reverse primer and T7 primer (Stratagene Inc., La Jolla, CA) were used as sequence primers. In addition different *PERI* gene-specific 17 basepairs oligonucleotides were generated to obtain overlapping sequences.

For analysis of the DNA and amino acid sequence the PCGENE-program release 6.5 (IntelliGenetics, Mountain View, CA) was used.

**Northern Hybridization, 3' End Mapping and cDNA Isolation**

Poly(A)^+ RNA was isolated from WT *H. polymorpha* grown in methanol-limited continuous culture as described by Brüningberg et al. (1989). Poly(A)^+ RNA was fractionated on formaldehyde-1% agarose gels and transferred onto nitrocellulose according to Sambrook et al. (1989). As probes for Northern analysis the 3-kb BamH1-BglI and the 630-bp Asp718-HinflI fragments were labeled using the random primed labeling kit of Boehringer Mannheim (Indianapolis, IN). Hybridization was performed in 0.5 M NaHPO4-buffer (pH 7.2) supplemented with 1 mM EDTA and 7% (wt/vol) SDS at 65°C for 16-18 h. After hybridization, filters were washed once in 2× SSC (Sambrook et al., 1989), 0.5% SDS for 10 min at 65°C, and three times in 1× SSC, 0.5% SDS for 10 min at room temperature. Finally the SDS was removed by two short washes with 2× SSC at room temperature. For determination of the 3' end of the PERI gene the Rapid Amplification of cDNA Ends (RACE) protocol (Frohman et al., 1988) was used as described by Frohman (1989). Poly(A)^+ RNA of *H. polymorpha* was reverse transcribed into CDNA using AMV Reverse Transcriptase (Boehringer Mannheim Corp.) and a hybrid dT17-adapter primer: 5' AACACGCTATGCCACCGCAGCCTTTTTTTTTTTTTTTTTTTTTT.
Table I. Strains and Plasmids Used in This Study

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3', containing a NotI restriction site. PCR amplification of the 3' end of PERI gene using CETUS TAQ-polymerase (The Perkin-Elmer Corp., Norwalk, CT) was performed with the adapter primer: AACGCTATGACCATG and the PERI gene specific primers B: 5' CGTCCACTACCCCAAGC3' (position 137-153; see Fig. 2) or D: 5' TGAGCATCCAAATCCG 3' (position 902-918; see Fig. 2). After amplification, the specific cDNA fragment obtained with primer D was subcloned in pBSII KS' by digestion with SalI and NotI.

For isolation of a genomic DNA fragment comprising the 3' end of the PERI gene, chromosomal DNA was digested with HindIII, fractionated on a 1% agarose gel and transferred onto nitrocellulose according to Sambrook et al. (1989). As probe for Southern analysis the 670-bp HindIII-BglII fragment was used. Labeling and hybridization was performed as described for Northern blotting. Fragments ranging from 2.5 to 5 kb were subsequently isolated from agarose gel and ligated in phosphatase-treated HindIII-digested pBSII KS'. The plasmid with the genomic DNA fragment comprising the 3' end was identified by E. coli colony hybridization using the 670-bp HindIII-BglII fragment as probe (Sambrook et al., 1989).

PERI1 Disruption

For disruption of the WT PERI gene, the LEU2 gene of Candida albicans (Gift of Dr. E. Berardi, Ancona, Italy) was blunt-ligated between the Klenow-treated Asp718 and HindIII sites of the complementing 3-kb fragment in pBSII KS'. Selection for LEU2 insertion was performed with E. coli C600 grown on minimal M9 medium supplied with 40 μg/ml thiamine and threonine (Sambrook et al., 1989). The LEU2-containing insert was subsequently released from pBSII KS' by digestion with BamHI and BglII and linearly transformed to a leu1.1 ura3 H. polymorpha strain by electrotransformation (Faber et al., 1994). Leucinotropic strains were screened for their ability to grow on methanol by replica-plating on YNM agar plates. Methanol utilization-deficient (Mu~) strains were selected, and the resulting diploids checked for complementation. In addition, Mu+ strains were mated with different auxotrophic H. polymorpha strains and the resulting diploids checked for complementation. In addition, Mu+ strains were mated with different auxotrophic H. polymorpha strains and the resulting diploids checked for complementation. Integration was confirmed by Southern blot analysis of chromosomal DNA using the ECL direct nucleic acid labeling and detection system of Amersham Corp. (Arlington Heights, Ill.).

Generation of Antisera

For expression and purification of PERIp the Protein Fusion and Purification System supplied by New England Biolabs (Beverly, MA) was used. A Asp718-PstI (pBSII KS') fragment was subcloned in frame behind the malE gene into the pMAL-C2 vector digested with EcoRI and PstI, after Klenow-treatment of the Asp718 and EcoRI sites and transformed to E. coli strain DH5α. Expression of the maltose-binding protein (MBP)-PERI hybrid protein under control of P_tac was induced by addition of 0.03 mM isopropyl-β-D-thiogalactopyranoside to exponentially growing cultures. Purification of the hybrid protein, cleavage of the protein with factor Xa and the final purification of the PERIp was according to the manual of New England Biolabs, except that in all steps 0.25% Tween 20 was added to the buffer solutions.

Polyclonal antibodies against the purified protein were raised in rabbit.

Cell Fractionation

For cell fractionation, protoplasts were prepared and subsequently homogenized as described by Douma et al. (1985). The homogenate was subjected to differential centrifugation (6,500 g for 10 min, followed by 12,000 g for 10 min and 30,000 g for 30 min). The 30,000-g pellet (P3) and corresponding supernatant (S3) were used for biochemical analysis. In addition P3 pellets were fractionated on a discontinuous sucrose gradient as described (Douma et al., 1985).

Purified peroxisosomal fractions were separated in solubile matrix and membrane-associated proteins after lysis in 20 mM triethanolamine buffer, pH 7.8, followed by centrifugation as described by Sutler et al. (1993a).

Biochemical Methods

Crude extracts were prepared as described before (Waterham et al., 1992a). Enzyme activities of alcohol oxidase (Verduyn et al., 1984), catalase (Lück, 1963), formaldehyde dehydrogenase (van Dijken et al., 1976) and cytochrome c oxidase (Douma et al., 1985) were assayed by established procedures. β-Lactamase activity was measured spectrophotometrically at 486 nm in 100 mM potassium phosphate buffer, pH 7.0, using nitrocefin (final concentration of 0.025 mg/ml, Becton Dickinson, Eten, Leur, The Netherlands) as substrate. Protein concentrations were determined with the Biorad protein assay kit using bovine serum albumin as standard. SDS-PAGE electrophoresis was performed as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250. Western blotting experiments were performed with the ECL Western blot analysis system (Amer sham Corp.). Transfer of proteins onto nitrocellulose after SDS-PAGE electrophoresis using a semi-dry electroph blotter was according to Kyhse-Andersen (1984).

Expression of the PERI Gene

Expression of the PERI gene in WT H. polymorpha cells under various growth conditions was studied by Northern blot analysis of total RNA isolated from the cells and by measuring β-lactamase activity as reporter for the PERI promoter (P_PERI) induction. For the latter approach WT β-lactamase (see below) was cloned in frame behind PPERI using the unique Asp718 site. The expression unit was subcloned in pHIPX4 and trans-
formed to a leul.1 H. polymorpha strain. The transformant was grown in batch cultures on selective mineral media supplemented with various carbon and nitrogen sources; cells were harvested at OD₆₆₀ = 2 for biochemical analysis.

**Targeting Experiments**

For targeting studies the β-lactamase gene of *E. coli* was used as reporter protein (Sutcliffe, 1978). This gene was cloned behind *P₁₆₁₈* and the initial 47 base pairs of the PERI gene, using the HaeIII site at position 47 (Fig. 2), resulting in a hybrid protein containing the amino-terminal 16 amino acids of PERIprec preceding β-lactamase protein expressed under control of the P₆₁₈. In addition, the final 27 base pairs of the PERI gene were cloned behind the modified β-lactamase gene using the HindIII site at position 1923 (see Fig. 2), and expressed under control of the P₂₃, resulting in a hybrid protein containing β-lactamase in front of the carboxy-terminal 9 amino acids of the PERI. All β-lactamase constructs were cloned in the *E. coli*--H. polymorpha shuttle-vector pHPX4 and transformed to a leul.1 H. polymorpha strain.

A deletion of the amino acids HKLGRQG (position 6-12) in the amino terminus of the PERI gene was introduced by restriction with Asp718 and Ncol, followed by Klenow-treatment and self-ligation (Fig. 2). The carboxy-terminal AKL tripeptide of the PERI was specifically removed by PCR, introducing a stop codon at position 1942-1944 using as primer 5′-CCGTTAACTTATTTTTCCTCACTCTCGTTGAC 3′. The protein containing β-lactamase in front of the carboxy-terminal 9 amino acids of the PERI were detected in the *E. coli*--H. polymorpha complementation group (strain perl-124/2D) using a *H. polymorpha* genomic DNA library. As reported before, this mutant belongs to a class of peroxisomal protein import mutants which are characterized by the presence of several small peroxisomes, although the bulk of the peroxisomal matrix protein resides in the cytosol (Pim⁻ phenotype, Fig. 1 B; Waterham et al., 1992b). Transformants were screened for leucine prototrophy and the ability to grow on methanol (Mut⁺-pheno type). Subsequently, one Mut⁺ transformant was isolated and further characterized. From this complemented strain three different plasmids with different inserts (6.6, 3.2, and 3 kb) were recovered in *E. coli*; upon retransformation only the plasmid with 3-kb insert could functionally complement the original perl-124/2D mutant. The presence of normal peroxisomes in the complemented mutant was confirmed by electron microscopy (Fig. 1 C).

Northern blot analysis using poly(A)⁺ RNA of methanol-grown *H. polymorpha* and the 3-kb insert as a probe, revealed a transcript of 2.2 kb (not shown); an identical transcript was also detected using the 3′ half of the insert (Asp718-BglII fragment) which indicates that at least part of the gene was present on the latter fragment.

Sequence analysis (see below) and additional Northern blotting, using small segments of the 3′ end of the 3-kb complementing fragment as probe, indicated that the fragment did not contain the entire PERI gene. This was confirmed by determination of the 3′ end of the PERI transcript using the RACE protocol. Two different cDNA fragments of 1.8 and 1.1 kb were amplified using the two PERI gene-specific primers B and D (see Materials and Methods), demonstrating that the complementing 3-kb fragment lacked 300 bp of the 3′ end of the PERI gene (not shown). Using part of the amplified cDNA fragments as probe an additional 3.7-kb HindIII genomic DNA fragment comprising the 3′ end of the PERI gene was subsequently isolated.

**Sequence Analysis of PERI**

Sequencing of the initially isolated 3-kb complementing DNA fragment revealed a large open reading frame (ORF) starting 11 bp in front of a unique Asp718 site and running out of the fragment without ending in a stop codon (Fig. 2). This indicated that not the entire PERI gene was cloned, thus confirming the data presented above. The ORF on this 3-kb genomic fragment only encodes 535 amino acids while vector PHRP2, used for the construction of the genomic DNA library, encodes an additional 88 amino acids, which results in a protein with a calculated total molecular mass of 70,616 kDa.

Sequencing of the cloned PCR amplified 1.1-kb cDNA fragment and of a part of the genomic HindIII fragment re-
revealed an additional stretch of 345 bp which belongs to the \textit{PERI-ORF} and encodes 115 amino acids. Therefore, the entire \textit{PERI} gene contains an ORF of 1,950 kb (Fig. 2). Several observations indicated that the first ATG of the ORF is the translation initiation site. Firstly, in front of this ATG several promoter elements were identified, like two putative TATA-elements, noted between positions -101 and -83, and the sequence CAAG, which often defines transcription initiation in yeast (Dobson et al., 1982), at position -34. Secondly, the large spacing between the observed promoter elements and the second ATG of the ORF (position 316) together with the possibility to amplify a cDNA fragment using the gene-specific primer B (position 137-153) make this second ATG unlikely to serve as translation initiation site. Furthermore, the size of the encoded protein determined by Western blotting (~70 kD; see below) approximates the calculated molecular mass when translation initiates at the first ATG. Initiation at the second ATG would result in a predicted molecular mass of 61.5 kD. The polyadenylation site was identified at position 1978 by sequencing of the cDNA fragments; the TAG codon in addition is part of the sequence TAGT(N)TTT which might serve as a polyadenylation signal in yeast (Zaret and Sherman, 1982). A yeast consensus sequence TACTAAC (Langford et al., 1984) for intron-splicing was not detected; the absence of introns was confirmed by the sizes of the two PCR amplified cDNA fragments obtained with the two different \textit{PERI} gene-specific primers.

The entire \textit{PERI} gene thus encodes a protein of 650 amino acids with a calculated molecular mass of 74 kD. Hydropathy analysis (Kyte and Doolittle, 1982) did not reveal any membrane-spanning segments although the protein is predicted to be rather hydrophobic. The amino acid sequence contains the extreme carboxy-terminal tripeptide AKL, a
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Figure 2. Nucleotide sequence and deduced primary sequence of the PERI gene. Some relevant restriction sites used for the different constructions are indicated above the nucleotide sequence. The initial complementing 3-kb fragment ended at the HindIII (H) site at position 1607. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z30206.

Figure 3. Disruption of the PERI gene. (a) The LEU2 gene of C. albicans was inserted between the Asp718 (A) and the HindIII (H) site of the coding region of PERI. Subsequently, the obtained construct was introduced into the genome by homologous integration. (b) The correct integration was confirmed by Southern blotting of genomic DNA cut with Sau3A (S) and probed with a BamH1 (B)-BglII (G) DNA fragment as indicated. Lane 1, genomic DNA isolated from WT H. polymorpha; lane 2, genomic DNA isolated from the disruption mutant.
variant of the PTSI which has been shown to serve as a peroxisomal targeting signal in PMP20 from *Candida boidinii* (Gould et al., 1989). An intriguing observation is that the amino terminus of the PER1 protein (PERlp) shows strong similarity to the consensus sequence RlxR/H/QL proposed for PTS2 (de Hoop and AB, 1992). In the PER1 sequence the arginine residue of the PTS2 consensus sequence is substituted by the similar residue lysine. Besides these two putative peroxisomal targeting signals, no functional sites were detected using the PROSITE software of the PC/GENE program. A search in different protein databases did not reveal any significant sequence similarity of PERlp to other proteins. However, recently the *Pastoris pastoris* PER3 gene was cloned by functional complementation of one of the *P. pastoris* per mutants (Liu et al., 1992). The PER3 protein shows ~60% similarity to PERlp (J. M. Cregg, unpublished results).

**Construction and Characterization of PER1 Disruption Mutants**

In order to confirm that the cloned complementing fragment indeed represents the *PER1* gene, a gene disruption was performed (Fig. 3 A); 35% of the leucine prototrophic transformants obtained appeared to be unable to utilize methanol (Mut− phenotype). Southern blot analysis performed with one randomly chosen Mut− transformant (*perl::LEU2*) indicated a correctly targeted chromosomal integration (Fig. 3 B). After mating of the *perl::LEU2* strain with auxotrophic WT strains to perform random spore analysis, a complete cosegregation of the Mut− phenotype and the *LEU2* gene was observed in all cases. In addition, diploids obtained after crossing of the *perl::LEU2* strain with the original mutant *perl-124/2D*, displayed the Mut− phenotype. These results demonstrate a correctly targeted integration of the *LEU2* gene in the *PER1* locus and therefore prove that the authentic *PER1* gene has been cloned. The *perl::LEU2* disruption mutant could only be functionally complemented by the entire *PER1* gene; after transformation with the initially isolated 3-kb genomic fragment, which encodes PERlp without its carboxy-terminal 115 amino acids (Fig. 2), the cells remained unable to grow on methanol.

Electron microscopy revealed that, after incubation of cells of the *perl::LEU2* disruption mutant on methanol, normal peroxisomes were lacking and the peroxisomal matrix proteins were localized in the cytosol (Fig. 4, A and B). However, several small vesicular structures were observed, which were absent in glucose-grown cells and therefore might represent peroxisomal remnants (Fig. 4, A, C, and D). Attempts to demonstrate the presence of alcohol oxidase protein (by immunocytochemistry) or activity (by the very sensitive *Ce*3 method) failed (results not shown). Therefore, matrix proteins are most probably lacking from these structures. It should be emphasized, that these structures however basically differ from the small protein–lipid aggregates, composed of mainly peroxisomal membrane proteins, which are observed in other peroxisome-deficient mutants of *H. polymorpha* (Sulter et al., 1993b; Waterham et

![Figure 4. Morphology of the *perl::LEU2* disruption mutant grown in a continuous culture on a glucose/methanol mixture (A–C) or incubated on methanol in batch culture (D). The mutant displays large cytosolic crystalloids (A, *), composed of alcohol oxidase protein (B, glutaraldehyde/OsO4). In these cells several vesicular structures were observed (A, C, and D, KMnO4) which might represent peroxisomal remnants. N, nucleus. Bar, 0.5 μm.](image-url)
Like all *H. polymorpha* Per<sup>-</sup> mutants studied so far (Sulter et al., 1990, 1991), the perI::LEU2 disruption mutant grew well on various compounds, the metabolism of which are mediated by peroxisomal matrix proteins (e.g., ethanol, d-alanine and methylamine). Also on these substrates normal peroxisomes were lacking and the matrix proteins were localized in the cytosol which indicates that PERlp is essential for peroxisome biogenesis in general.

In order to further analyze the nature of the vesicular structures cells of the perI::LEU2 disruption mutant, grown in continuous culture on a glucose/methanol mixture (Fig. 4, A and C), were fractionated by differential and sucrose density centrifugation. Protein analysis of the different fractions obtained from the sucrose gradient revealed the presence of one major protein peak at 43.5% sucrose (Fig. 5 a, fraction 25) and a small peak at 49% sucrose (Fig. 5 a, fraction 11). As expected from electron microscopy, the majority of the peroxisomal matrix proteins alcohol oxidase and catalase were soluble after differential centrifugation and thus a distinct peroxisomal peak fraction was not observed when these proteins were considered as reporter proteins (Fig. 5 b, intact WT peroxisomes usually fractionate at 53% sucrose). The minor alcohol oxidase protein peak, which fractionated on top of the 60–65% sucrose layer (Fig. 5 b), most probably represents remnants of the alcohol oxidase crystalloids which were present in the cells (Fig. 4 A). The protein peak fraction at 43.5% sucrose mainly consists of mitochondria (Fig. 5 c).

The protein contents of fraction 11 was analyzed by SDS-PAGE electrophoresis and compared to purified peroxisomes isolated from methanol-grown WT cells. As is evident from Fig. 6 a, fraction 11 clearly has several proteins in common with WT peroxisomes; furthermore, Western blotting experiments using specific antibodies against the peroxisomal membrane protein PER8 (Tan, X., H. R. Waterham, M. Veenhuis, and J. M. Cregg, manuscript in preparation) clearly demonstrate the presence of this protein in fraction 11 (Fig. 6 b). These results indicate that this fraction contains components of peroxisomal membranes. The low levels of alcohol oxidase protein in this fraction is probably caused by the mentioned contamination with the alcohol oxidase crystalloids.

### Expression of the PERI Gene

To obtain insight in the significance of PERlp in peroxisome biogenesis, the expression of the protein was studied during growth of WT cells in media containing various carbon and nitrogen sources, known to induce peroxisome proliferation.

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**Figure 5.** Subcellular fractionation of the *perI::LEU2* disruption mutant grown in a glucose/methanol limited continuous culture. The 30,000-g pellet obtained after differential centrifugation of homogenized protoplasts was fractionated on a sucrose gradient and divided in different fractions (see Materials and Methods). (a) Protein concentrations (mg/ml) and sucrose density (% wt/wt). (b) Catalase and alcohol oxidase activities. Catalase activity is expressed as Δ 260 nm/min/ml, alcohol oxidase as U/ml. (c) Cytochrome c oxidase and formaldehyde dehydrogenase activities (U/ml).

**Figure 6.** SDS-PAGE electrophoresis and Western blot analysis of fraction 11 obtained by cell fractionation of the *perI::LEU2* disruption mutant (Fig. 5), compared to purified peroxisomes of methanol-grown WT *H. polymorpha*. Proteins were separated on a 12.5% polyacrylamide gel and visualized by Coomassie staining (a) or blotted for Western analysis using specific antibodies against PER8 protein (b). Lane 1, purified WT peroxisomes; lane 2, soluble fraction of purified WT peroxisomes, lysed in 20 mM triethanolamine (TEA) buffer, pH 7.8; lane 3; pelletable fraction of purified WT peroxisomes, lysed in 20 mM TEA, pH 7.8; lane 4, fraction 11 obtained from sucrose gradient in Fig. 5.
which indicates that PERlp is a protein of low abundance in H. polymorpha. Northern blot analysis of 25 μg total RNA isolated from cells grown in batch culture on glucose, methanol, ethanol, glycerol, d-alanine, or methyamine, using the PERI gene as probe, only revealed a rather weak signal in methanol-grown cells; in all other samples no signal was detected (not shown). For this reason we decided to study the PPERI-driven synthesis of bacterial β-lactamase, thus taking advantage of the sensitive β-lactamase activity assay. The results shown in Fig. 7 indicate that PPERI is active under all growth conditions tested. Compared to glucose/ammoniumsulphate-grown cells, a small increase in activity was observed when ammoniumsulphate was replaced by d-alanine or methyamine as nitrogen source. PPERI activity was further enhanced when cells were grown on carbon sources, the metabolism of which require microbody enzymes. As was expected, maximal activity was observed during growth of cells on methanol. The observed regulation of PPERI differs from the induction of the promoters of alcohol oxidase and catalase, as is indicated by the respective enzyme activities, detected in the same cells (Fig. 7). Since we used an autonomous replicating plasmid in these experiments, control experiments were performed using the same vector containing the β-lactamase gene expressed under control of the PMox. In glucose- or ethanol-grown cells no β-lactamase activity was detected, which indicates that carbon catabolite repression of PMox (Veenhuis and Harder, 1991) operates efficiently, even in the case of replicating plasmids. In methanol-grown transformants the PPERI driven activity was further enhanced when cells were grown on carbon sources, the metabolism of which require microbody enzymes. As was expected, maximal activity was observed during growth of cells on methanol. The observed regulation of PPERI differs from the induction of the promoters of alcohol oxidase and catalase, as is indicated by the respective enzyme activities, detected in the same cells (Fig. 7). Since we used an autonomous replicating plasmid in these experiments, control experiments were performed using the same vector containing the β-lactamase gene expressed under control of the PMox. In glucose- or ethanol-grown cells no β-lactamase activity was detected, which indicates that carbon catabolite repression of PMox (Veenhuis and Harder, 1991) operates efficiently, even in the case of replicating plasmids. In methanol-grown transformants the PPERI driven activity (1.5 units) is low compared to the activity resulting from expression behind PMox (100.4 units), which indicates that PERIp is a protein of low abundance in H. polymorpha.

The PERI Gene Product Is a Peroxisomal Matrix Protein

Polyclonal antibodies raised against PERIp were used in Western blotting experiments to determine its subcellular localization. The size of the protein, which was specifically recognized by these antibodies, is ~70 kD which is in good agreement with the calculated molecular mass and the size of PERIp observed after cleavage with factor Xa of the maltose-binding protein-PERIp fusion protein synthesized in E. coli (not shown). In crude extracts prepared of methanol-grown cells of WT H. polymorpha PERIp could hardly be detected, again indicating that PERIp is a low abundant protein. However, when these cells were fractionated by differential centrifugation and subsequent sucrose gradient centrifugation, PERIp was specifically detected in highly purified peroxisomes (Fig. 8 and 9). The occasionally coexisting of alcohol oxidase in the Western blots, decorated with anti-PER19, most probably is not specific but due to the excessive amounts of alcohol oxidase proteins present in peroxisomes of WT H. polymorpha. When subsequently purified peroxisomes were separated in a soluble matrix and a membrane protein fraction, PERIp was exclusively detected in the soluble fraction, indicating that PERIp is a component of the peroxisomal matrix (Fig. 8). As expected, PERIp could not be detected in fraction 11 of the sucrose gradient obtained from the per1::LEU2 disruption mutant (not shown).

The intra-peroxisomal localization of PERIp in WT H. polymorpha was also studied by immunocytochemistry, performed on thin sections of transformants in which the PERI gene was expressed behind the PMox. In these cells labeling was predominantly present at edge of the peroxisomes (Fig. 1 D). We have interpreted our combined data in that Perlp...
Localization of PERlp. Equal amounts of purified peroxisomes (lane 1) and mitochondria (lane 2) obtained after differential and subsequent sucrose density centrifugation of methanol-grown WT cells of *H. polymorpha* (compare Fig. 8, fraction 7 and fraction 28, respectively) were fractionated on a 7.5% SDS-polyacrylamide gel and blotted for Western analysis using specific antibodies raised against PERlp (40 μg protein/lane). The intra-peroxisomal localization was evident after Western analysis of pelletable (lane 3) and soluble (lane 4) fractions obtained from 40 μg purified WT peroxisomes (Fig. 8, fraction 8) after lysis in 20 mM triethanolamine buffer, pH 7.8.

is not included in the alcohol oxidase crystalloids but instead present in the small zone between the crystalloid and the surrounding membrane, comparable to for instance catalase protein in identically grown cells (Keizer-Gunnink et al., 1992).

**PER1 Protein Contains the Two Conserved Peroxisomal Targeting Signals PTS1 and PTS2**

As indicated above, PERlp contains both the carboxy-terminal PTS1 (AKL) and the amino-terminal PTS2 (KLxQL) peroxisomal targeting consensus sequences. In order to test whether these putative PTS sequences are indeed functional in *H. polymorpha*, we studied whether they were capable of targeting bacterial β-lactamase protein to peroxisomes. Previously, this protein was successfully used to identify the PTS1 targeting signals of *H. polymorpha* alcohol oxidase and dihydroxyacetone synthase (Hansen et al., 1992). Recent experiments showed that β-lactamase was also suitable for targeting studies with the PTS2 signal (Faber, K. N., P. Haima, C. Gietl, W. Harder, G. AB, and M. Veenhuis, manuscript submitted for publication). The primary sequences of the hybrid proteins used for these experiments are detailed in Table II. Immunocytochemical experiments, using antibodies against β-lactamase, showed that both the amino-terminal 16 amino acids, containing the PTS2 consensus sequence, as well as the 9 carboxy-terminal amino acids, containing the PTS1 sequence, were capable of targeting β-lactamase to peroxisomes (Fig. 10).

The functionality of the carboxy- and amino-terminal targeting signals was furthermore analyzed by transformation of the *perl::LEU2* disruption mutant with different mutated versions of the *PER1* gene, specifically mutagenized in the PTS consensus sequences (see Table II). The mutant could still be functionally complemented by the *PER1* constructs in which either the whole PTS1 sequence (AKL) or a major part of the PTS2 consensus sequence (HKLGRQG) was deleted. In both cases the transformed cells were able to grow on methanol and contained several peroxisomes.

**Discussion**

We have identified and characterized a peroxisomal protein essential for the biogenesis of peroxisomes in the methylotrophic yeast *H. polymorpha*. The *PER1* gene coding for this protein was cloned by functional complementation of a *H. polymorpha* peroxisomal protein import mutant (*Pim* phenotype; Waterham et al., 1992b), using a *H. polymorpha* genomic DNA library. The gene comprises an ORF which encodes a polypeptide of 650 amino acids with a calculated molecular mass of 74 kD.

Interestingly, the original complementing genomic fragment lacked the 3' end of the *PER1* gene, which encodes the 114 carboxy-terminal amino acids. One possible explanation for this rather surprising result could be that a recombination event took place between the complementing fragment and the mutant *perl* allele, thus giving rise to a WT *PER1* gene.
in the genome. Several observations, however, indicated that this was not the case. First, after introduction of the incomplete gene on the pHPR2 vector in the original perl-124/2D mutant, all leucine prototrophic strains were complemented for growth on methanol. Second, the pHPR2 vector was readily recovered from the complemented mutant yeast strain by electrottransformation of E. coli with total yeast DNA. Third, the complemented mutant could be forced to lose its plasmid by growth on nonselective media, resulting again in a Mut- leu strain. These results, together with the fact that this fragment could not functionally complement the perl::LEU2 disruption mutant, indicate that PERlp probably functions as a multimeric protein containing at least two PER1 subunits; in the original perl-124/2D strain one mutated subunit is apparently able to functionally complement the other.

So far, PERlp is the only peroxisomal protein described which contains both a PTS1 and a PTS2 motif although other peroxisomal matrix proteins are reported to contain several putative (internal) targeting sequences, like Candida tropicalis acyl CoA oxidase (Small et al., 1988) and S. cerevisiae catalase (Kragler et al., 1993). The complementation experiments with the perl::LEU2 disruption mutant using different mutated PER1 genes, combined with the results obtained in the targeting experiments using β-lactamase as a reporter protein, suggest that both PTS motifs might function in vivo. Additional evidence for the in vivo functionality of the PTS2 sequence was obtained by studying the location of PERlp in a PER3 disruption mutant of H. polymorpha. This mutant is impaired in the import of PTS1 targeted peroxisomal proteins (similar to the Pichia pastoris PAS8; McCollum et al., 1993), whereas PTS2 containing proteins are correctly imported into the present small peroxisomes (van de Klei, I. J., G. J. Swaving, R. H. Hilbrands, H. R. Waterham, V. Titorenko, J. M. Cregg, W. Harder, and M. Veenhuis, manuscript in preparation). Western blot analysis of purified peroxisomes from the PER3 disruption mutant revealed that PERlp was localized in these organelles (H. R. Waterham and G. J. Swaving, unpublished results).

The biochemical studies showed that PERlp is a protein of low abundance located in the peroxisomal matrix. At present, PERlp is the first matrix protein shown to be essential for peroxisome biogenesis. Deletion of other major peroxisomal matrix proteins in H. polymorpha, like alcohol oxidase, catalase or amine oxidase, results in a decrease in size of peroxisomes, but never affects the biogenesis of the organelles (Titorenko et al., 1993). The PER8 gene was recently cloned and encodes an integral peroxisomal membrane protein (Tan, X., H. R. Waterham, M. Veenhuis, and J. M. Cregg, unpublished results). Interestingly, when the PER8 gene was overexpressed behind the Pmox a strongly enhanced proliferation of peroxisomes occurred in methanol-grown cells, indicating that PER8p plays an important role in the regulation of peroxisome proliferation. Our observations that in the perl::LEU2 disruption mutant PER8p was present in the vesicular structures and that multiplication of these structures occurred but no import of matrix proteins, suggest that PERlp and PER8p may coordinate the development of new peroxisome import competent peroxisomes by fission from preexisting organelles.

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