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, PER1p. 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 PER1p 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. PER1p 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* PER1p is a protein of low abundance which was demonstrated to be localized in the peroxisomal matrix. Our results suggest that the import of PER1p into peroxisomes is a prerequisite for the import of additional matrix proteins and we suggest a regulatory function of PER1p 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)[1] 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 (Didion and Roggenkamp, 1992), SKF (Kragler et al., 1993), ARF, and NKL (Hansen et al., 1992) which act as a PTS1 in different yeast species. Subsequently, a second peroxiso-

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1. *Abbreviations used in this paper*: MBP, maltose-binding protein; ORF, open reading frame; PTS, peroxisomal-targeting signal; WT, wild-type.

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 RLx_5Q/HL 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., 1992*b*), *P. pastoris* (Gould et al., 1992; 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 *PER1* 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% [vol/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 Per1 Mutants

The isolation, back-crossing, complementation analysis, gene mapping, and phenotype characterization of various *H. polymorpha per* mutants, including *per1* mutants, are detailed in previous papers (Veenhuis, 1992; Waterham et al., 1992*b*; Titorenko et al., 1993).

Construction of a H. polymorpha genomic DNA Library

Chromosomal DNA from YPD-grown wild-type (WT) *H. polymorpha* harvested at OD_{660}-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 *Sau*3A, 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 5% 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 phosphatase-treated unique *Bam*HI site of the autonomous replicating *E. coli–H. polymorpha* shuttle vectors pHRP1 and pHRP2. Ligation mixtures were transformed to *E. coli* MC1061 by electrotransformation (Faber et al., 1992). After transformation $\sim 2 \times 10^4$ 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 2×10^7 bp).

Cloning of the PER1 Gene

To isolate the *Per1* gene, a three-times backcrossed strain of mutant *per1-124/2D* (Waterham et al., 1992*b*) was transformed with the constructed genomic DNA library (Faber et al., 1992). After 4–5 d growth on YND-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 *per1-124/2D* 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 polymerase-treated *Nhe*I-*Sph*I fragment (both sites exclusively present in pHRP2-vector) in the unique *Sma*I-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 Deaza^{T7} sequencing^{TM} kit of Pharmacia LKB Biotechnology 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 *PER1* 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 Bruinenberg 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 *Bam*HI-*Bgl*I and the 630-bp *Asp*718-*Hind*III fragments were labeled using the random primed labeling kit of Boehringer Mannheim (Indianapolis, IN). Hybridization was performed in 0.5 M $NaHPO_4$-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 *PER1* gene the Rapid Amplification of cDNA Ends (RACE) protocol (Frohman et al., 1988) was used as described by Frohman (1990) with slight modifications. 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' AACAGCTATGACCATGCGGCCGCTTTTTTTTTTTTTTTT

Table I. Strains and Plasmids Used in This Study

Strain/plasmid	Relevant properties	Source or reference
H. polymorpha		
per1-124/2D	*leu1.1 per1*	Waterham et al., 1992*b*
per1::LEU2	*per1 ura3*	This study
CBS4732	WT	Central Bureau Schimmelcultures collection, The Netherlands
NCYC495	auxotrophic collection	Gleeson and Sudbery, 1988
E. coli		Sambrook et al., 1989
MC1061	*hsdR mcrB araD139 (araABC-leu)7679 lacX74 galU galK rpsL (Str^r)*	Sambrook et al., 1989
DH5α	*supE44 hsdR17 thi-1 recA1 endA1 gyrA relA1*	
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	Sambrook et al., 1989
Plasmids		
pHRP1/2	Ap^r, *LEU2*, *HARS1*	Faber et al., 1992
pHARS1	Ap^r, *URA3*, *HARS1*	Roggenkamp et al., 1986
pBluescriptIIKS$^+$	Ap^r	Stratagene Inc.
pMAL-C2	Ap^r, P_{tac}*malE*	New England Biolabs
pHIPX4	Km^r, *LEU2*	Gietl et al., 1994

3′, containing a *Not*I restriction site. PCR amplification of the 3′ end of *PER1* gene using CETUS TAQ-polymerase (The Perkin-Elmer Corp., Norwalk, CT) was performed with the adapter primer: AACAGCTATGACCATG and the *PER1* gene specific primers B: 5′ CGTCCACTACCCCAGAC 3′ (position 137–153; see Fig. 2) or D: 5′ TGAGCATCCCAAATCCG 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 *Sal*I and *Not*I.

For isolation of a genomic DNA fragment comprising the 3′ end of the *PER1* gene, chromosomal DNA was digested with *Hin*dIII, fractionated on a 1% agarose gel and transferred onto nitrocellulose according to Sambrook et al. (1989). As probe for Southern analysis the 670-bp *Hin*dIII-*Bgl*I 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 *Hin*dIII-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 *Hin*dIII-*Bgl*I fragment as probe (Sambrook et al., 1989).

PER1 Disruption

For disruption of the WT *PER1* gene, the *LEU2* gene of *Candida albicans* (Gift of Dr. E. Berardi, Ancona, Italy) was blunt-ligated between the Klenow-treated *Asp*718 and *Hin*dIII 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 *Bam*HI and *Bgl*I and linearly transformed to a *leu1.1 ura3 H. polymorpha* strain by electrotransformation (Faber et al., 1994). Leucine prototrophic strains were screened for their ability to grow on methanol by replica-plating on YNM agar plates. Methanol utilization-deficient (Mut$^-$) strains were selected, mated with *per1-124/2D* and the resulting diploids checked for complementation. In addition, Mut$^-$ strains were mated with different auxotrophic *H. polymorpha* NCYC 495 strains to determine the location of the genomic *LEU2* integration by random spore analysis. 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, IL).

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 *Asp*718-*Pst*I (pBSII KS$^+$) fragment was subcloned in frame behind the *mal*E gene into the pMAL-C2 vector digested with *Eco*RI and *Pst*I, after Klenow-treatment of the *Asp*718 and *Eco*RI sites and transformed to *E. coli* strain DH5α. Expression of the maltose-binding protein (MBP)-PER1 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 peroxisomal fractions were separated in soluble matrix and membrane-associated proteins after lysis in 20 mM triethanolamine buffer, pH 7.8, followed by centrifugation as described by Sulter et al. (1993*a*).

Biochemical Methods

Crude extracts were prepared as described before (Waterham et al., 1992*a*). 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 nitrocefine (final concentration of 0.025 mg/ml; Becton Dickinson, Etten, 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 (Amersham Corp.). Transfer of proteins onto nitrocellulose after SDS-PGE electrophoresis using a semi-dry electroblotter was according to Kyhse-Andersen (1984).

Expression of the PER1 Gene

Expression of the *PER1* 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 *PER1* promoter (P_{PER1}) induction. For the latter approach WT β-lactamase (see below) was cloned in frame behind P_{PER1} using the unique *Asp718* site. The expression unit was subcloned in pHIPX$_4$ and trans-

formed to a *leu1.1 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 OD_{663} = 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 P_{PER1} and the initial 47 base pairs of the *PER1* gene, using the *Hae*III site at position 47 (Fig. 2), resulting in a hybrid protein containing the amino-terminal 16 amino acids of PER1p preceding β-lactamase protein expressed under control of the P_{PER1}. In addition, the final 27 base pairs of the *PER1* gene were cloned behind the modified β-lactamase gene using the *Hin*dII site at position 1923 (see Fig. 2), and expressed under control of the P_{MOX}, resulting in a hybrid protein containing β-lactamase in front of the carboxy-terminal 9 amino acids of the PER1p. All β-lactamase constructs were cloned in the *E. coli–H. polymorpha* shuttle-vector pHIPX4 and transformed to a *leu1.1 H. polymorpha* strain.

A deletion of the amino acids HKLGRQG (position 6–12) in the amino terminus of the *PER1* gene was introduced by restriction with *Asp*718 and *Nar*I, followed by Klenow-treatment and self-ligation (Fig. 2). The carboxy-terminal AKL tripeptide of the PER1p was specifically removed by PCR, introducing a stop codon at position 1942–1944 using as primer 5′ CCGTTAACTTATTTTTCCTCACTCTCGTTGAC 3′. The *PER1* deletion constructs were cloned in the pHARS1 vector and tested for functional complementation of a *per1::LEU2* disruption mutant. Constructs were checked by restriction analysis and sequencing. The primary sequences of the resulting constructs are shown in Table II.

Electron Microscopy

Whole cells were fixed in 1.5% (wt/vol) $KMnO_4$ for 20 min at room temperature. Spheroplasts were fixed in 6% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 60 min at 0°C, followed by postfixation in a mixture of 0.5% (wt/vol) OsO_4 and 2.5% (wt/vol) $K_2Cr_2O_7$ in the cacodylate buffer for 90 min at 0°C. After dehydration in a graded ethanol series the samples were embedded in Epon 812; ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

For immunocytochemistry intact cells were fixed in either 3% (vol/vol) glutaraldehyde or 3% (vol/vol) formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 90 min at 0°C, dehydrated in a graded ethanol series and embedded in Unicryl. Immunolabeling was performed on ultrathin sections with specific antibodies and gold-coupled goat anti-rabbit antibodies by the method of Slot and Geuze (1984).

Miscellaneous DNA Techniques

Standard recombinant DNA techniques were performed essentially as described by Sambrook et al. (1989). Restriction and DNA-modifying enzymes were obtained from and used as recommended by the manufacturers (Boehringer Mannheim Corp., Pharmacia Diagnostics Inc. [Fairfield, NJ], and New England Biolabs).

Results

Cloning of the PER1 Gene

The *PER1* gene was cloned by functional complementation of one of the representative mutants of the *PER1* complementation group (strain *per1-124/2D*) 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., 1992*b*). Transformants were screened for leucine prototrophy and the ability to grow on methanol (Mut^+-phenotype). 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 *per1-124/2D* 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-BglI* 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 *PER1* gene. This was confirmed by determination of the 3′ end of the *PER1* transcript using the RACE protocol. Two different cDNA fragments of ~1.8 and 1.1 kb were amplified using the two *PER1* 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 *PER1* gene (not shown). Using part of the amplified cDNA fragments as probe an additional 3.7-kb *Hin*dIII genomic DNA fragment comprising the 3′ end of the *PER1* gene was subsequently isolated.

Sequence Analysis of PER1

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 *Asp*718 site and running out of the fragment without ending in a stop codon (Fig. 2). This indicated that not the entire *PER1* 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 kD.

Sequencing of the cloned PCR amplified 1.1-kb cDNA fragment and of a part of the genomic *Hin*dIII fragment re-

Table II. Primary Sequences of DNA Constructs Used for Targeting Studies

Construct	Promoter	Amino acid sequence
N^{16}-βLAC	P_{PER1}	M^1QPWYHKLGRQGRQLA^{16}*H*^{24}*PE-262aa-HW*^{286}*
βLAC-C^9	P_{MOX}	*MSGH*^{24}*PE-262aa-HW*^{286}*S*N^{642}ESQEKAKL^{650}*
per1-ΔHKLGRQG	P_{PER1}	M^1QPWY^{5}R^{13}QLAEQWQ-625aa-E^{646}KAKL^{650}*
per1-ΔAKL	P_{PER1}	M^1QPWYHKLGRQGRQLA^{16}-625aa-V^{641}NESQEK^{647}*

Roman, amino acid sequence of PER1p; numbers as indicated in Fig. 2.
Italics, amino acid sequence of β-lactamase; numbers correspond with WT β-lactamase (Sutcliffe, 1978).
*, carboxy terminus of resulting proteins.

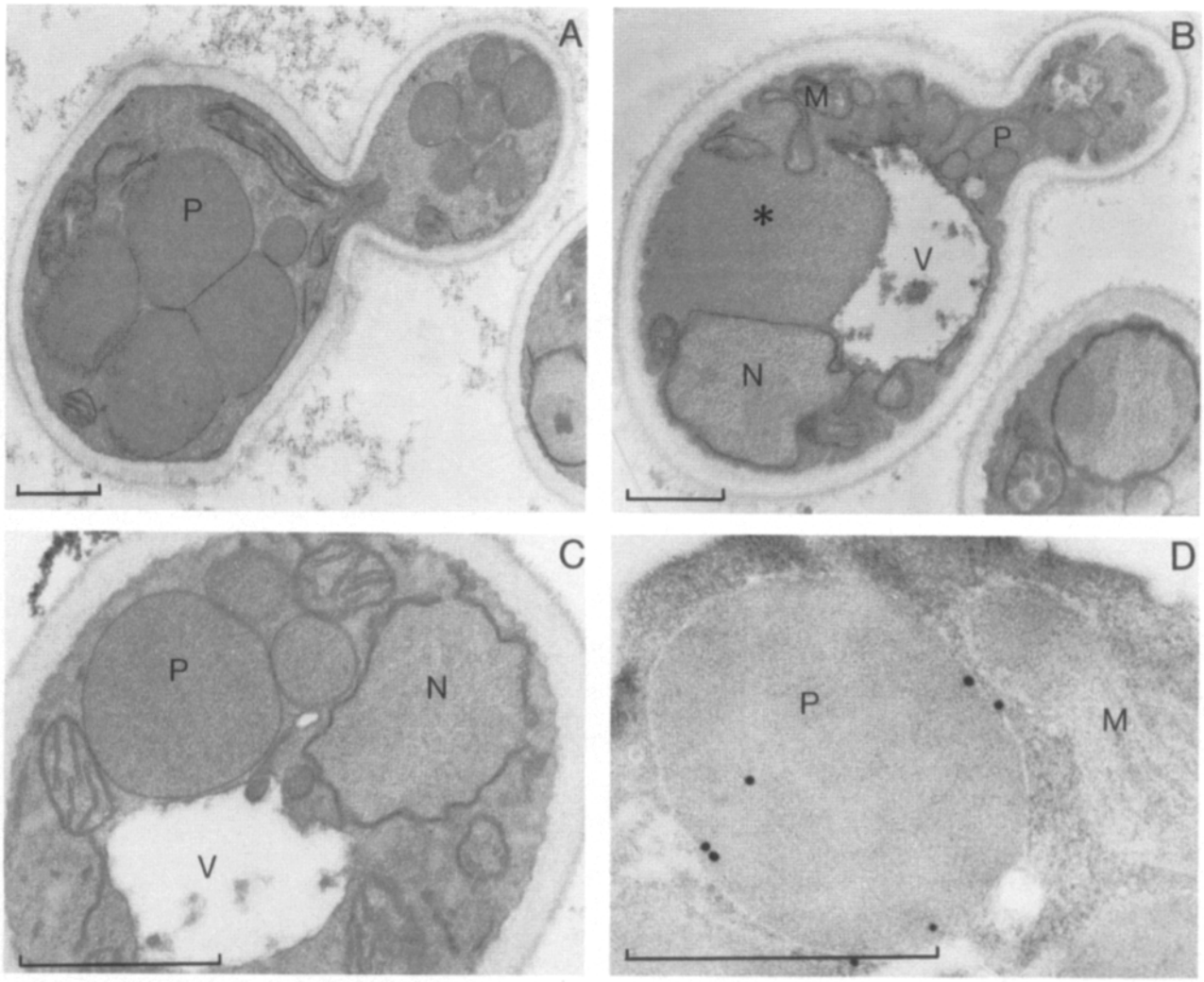

Figure 1. Overall morphology of methanol-grown WT cells of *H. polymorpha*, showing proliferation of peroxisomes (*A*, $KMnO_4$), compared to methanol-induced cells of the *perl-124/2D* mutant strain, showing a large cytosolic alcohol oxidase crystalloid (*) and several small peroxisomes (*B*, $KMnO_4$). Thin section through a methanol-grown cell of the functionally complemented *perl-124/2D* strain showing the presence of normal, intact peroxisomes (*C*, $KMnO_4$). Immunocytochemistry, using specific antibodies raised against PER1p revealed that PER1p is localized at the edge of peroxisomes (*D*). The rather low labeling intensity is significant for organelles of these growth conditions and most probably reflects the low abundance of the Perlp. *P*, peroxisome; *N*, nucleus; *V*, vacuole; *M*, mitochondrion. Bar, 0.5 μm.

vealed an additional stretch of 345 bp which belongs to the *PERI-ORF* and encodes 115 amino acids. Therefore, the entire *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 *PERI* gene-specific primers.

The entire *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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-215                                  CACAAACAGGGGTTTCCACTCAAAGATCGACACGGCAA

-177  GCGGAACGGCAATGAACACCGACGTCAACGCCGAGCTGATCGGAGATGGACAAACCCTG

-118  TGGGGAGCGAGGAGGACATTTAAATAAATAAAGTTAAATACTATTTCCAAGGACTACCG
           MluI
-59   ACGCGTCGTAAAATAGCCTAAAGATCAAGAAATAATTTATCTAGTTTCTTGTTCCTACC

                    Asp718                                  NarI
1     ATG CAG CCG TGG TAC CAT AAA CTG GGC AGG CAG GGG CGC CAA CTG
       M  Q  P  W  Y  H  K  L  G  R  Q  G  R  Q  L                15
           HaeIII
46    GCC GAG CAA TGG CAG ACT GAT GCA GAG CCG TGG GGC GTT GCT ACT
       A  E  Q  W  Q  T  D  A  E  P  W  G  V  A  T                30

91    CCG ACT CCT TTG GAT TAT CTG TTC GAC GAA TTA ACT GCT CCT AAA
       P  T  P  L  D  Y  L  F  D  E  L  T  A  P  K                45

136   CCG TCC ACT ACC CCA GAC AAA GTC CTA TCA TAT CTG GCA TAC TAC
       P  S  T  T  P  D  K  V  L  S  Y  L  A  Y  Y                60

181   TAT CCA AAA CTG AAA AAC GAG AAC AAT GTC GAG CTT TTG ACG CTC
       Y  P  K  L  K  N  E  N  N  V  E  L  L  T  L                75

226   TGT TTC CTT CGA TGT CCA CTT TTC TTC AAC GAT GCC CAG CTG GTG
       C  F  L  R  C  P  L  F  F  N  D  A  Q  L  V                90

271   TCG TTC AGC GAC AAC TAT CGC GTT ATC GAG TGC TTC AAG TAC ATC
       S  F  S  D  N  Y  R  V  I  E  C  F  K  Y  I               105

316   ATG GAC AAG AAG TTC CAG ATC AGC CAG CCG ACG CTG CCC TTT TAC
       M  D  K  K  F  Q  I  S  Q  P  T  L  P  F  Y               120

361   AGG TTC TAC AAC GCG CTG TTT GGA GCC CTT GCG AAG GTC GTC GCA
       R  F  Y  N  A  L  F  G  A  L  A  K  V  V  A               135

406   GAT AGC GCG TGC GCG TGG AAA GTG GTC CCC GTG GCT GTT GGG TGC
       D  S  A  C  A  W  K  V  V  P  V  A  V  G  C               150

451   TTG CTC TCT GTG GAC TCA AGA AAC GAC TAC GAC AGG TAT CCC GAG
       L  L  S  V  D  S  R  N  D  Y  D  R  Y  P  E               165

496   CAT TTT CAG CTC ATT GCA ACA GTA GAC GCC AAA CTC GTG GAC ATG
       H  F  Q  L  I  A  T  V  D  A  K  L  V  D  M               180

541   GCA GCA CAT ACG CTG GAA AGA AGC ATG GAC GGG CCG CTT TCA AAT
       A  A  H  T  L  E  R  S  M  D  G  P  L  S  N               195

586   GAT CTG CTA TGT CTC AAC GTG GTT GCC TTG TCC TGT GTC CAG GAC
       D  L  L  C  L  N  V  V  A  L  S  C  V  Q  D               210
     HindIII  SalI
631   AAG CTT GTC GAC TCT CAA CTA CTG CGA ATT TTG CGA GTG AGA TCC
       K  L  V  D  S  Q  L  L  R  I  L  R  V  R  S               225

676   GAC ATT TTG AAA ATT CTC ACA GAG CTC ACG TTC AAC TCG CCA TAC
       D  I  L  K  I  L  T  E  L  T  F  N  S  P  Y               240

721   GGT CTG GAT AAT GGT CGT CTT TTG ACA AAA AGC AAC GCA AAC ACC
       G  L  D  N  G  R  L  L  T  K  S  N  A  N  T               255

766   CCC ATC GTT GTC AGA CAC CTA AAT CGG ATC TCG TTC CTG TTC ACC
       P  I  V  V  R  H  L  N  R  I  S  F  L  F  T               270

811   AAG CTC ACG TCG ATC CAC CCA AAA ATC GTT TTG TTG GCG GAC GAT
       K  L  T  S  I  H  P  K  I  V  L  L  A  D  D               285

856   TTG GAT TTG ATT CTG AAC AGG ATT CAA ACG TTC AGC GAG AGC GTG
       L  D  L  I  L  N  R  I  Q  T  F  S  E  S  V               300

901   CTG AGC ATC CCA AAT CCG TCA GAA ACG CAA TGG AGT ACA CTA AGA
       L  S  I  P  N  P  S  E  T  Q  W  S  T  L  R               315

946   GTG GTA TTG TTT GCG CAG GTC ATG ATG TTT GAA GGA ATC ATG GCC
       V  V  L  F  A  Q  V  M  M  F  E  G  I  M  A               330

991   CGT TTC TTT CAA ATC AAC AAC CAT TCT CTC AAC AGC ACC GTC CTG
       R  F  F  Q  I  N  N  H  S  L  N  S  T  V  L               345

1036  CCC ACC CTG TGT AGG AAA ATC CTT ACA ACG CTG TTC AAC TTC AAT
       P  T  L  C  R  K  I  L  T  T  L  F  N  F  N               360
                 SalI
1081  TTT GTC GTC GAC AGA ATC GGC ACT GGC GGG TTC GAG AGC TAC AAT
       F  V  V  D  R  I  G  T  G  G  F  E  S  Y  N               375

1126  TTC GTC TAC GCG TCG TGT CTC AGC ACA CTC ACA AGC TAC GAC ATT
       F  V  Y  A  S  C  L  S  T  L  T  S  Y  D  I               390

1171  CCC ACG GCA GAA ACG CTC ATC AAA TGC TGG ACC AGC AGC GTA GCA
       P  T  A  E  T  L  I  K  C  W  T  S  S  V  A               405

1216  TTC AAG AAA GTG GAC AAT TCT GCT ACA GAG CGC GGG AAA CTG CTG
       F  K  K  V  D  N  S  A  T  E  R  G  K  L  L               420

1261  TTT GAT CTG CAA TTT ATC GAA AAT GTC GTC AAC TTG GTC TCC GAC
       F  D  L  Q  F  I  E  N  V  V  N  L  V  S  D               435

1306  AGC CTC AAG TTC GAG TTT ATC ATC CCC ATA GTC CAA GAC CTG ATA
       S  L  K  F  E  F  I  I  P  I  V  Q  D  L  I               450

1351  GGT AAT GCA CAA GAC CAG GCT GTC TTG GAG AGC GCA CAT TCA GTC
       G  N  A  Q  D  Q  A  V  L  E  S  A  H  S  V               465

1396  ATG CTT AAA TAC TTC ACC AGC GTC GAT ACC TAC AAC GAG GCC CAA
       M  L  K  Y  F  T  S  V  D  T  Y  N  E  A  Q               480

1441  TTG GTG GAC TAC ACG AAC AAC GTC AAA CAT GTT GGA GCG CAG CTG
       L  V  D  Y  T  N  N  V  K  H  V  G  A  Q  L               495
                 BglI
1486  ATC GAT TAC CTT ACG CTG TCC CTA GAC CAG TTT CCG GCC CGA CTG
       I  D  Y  L  T  L  S  L  D  Q  F  P  A  R  L               510
                 BglI
1531  TCG CTG AGC CAA GTC GGC ATT ATT GTC GAG ACA CTG GCC AAA ATC
       S  L  S  Q  V  G  I  I  V  E  T  L  A  K  I               525
                    KspI                    Sau3A
1576  ACG TTT CCG GAC ACC GCG GTG CAC GAG TGC GAT CCA GAA CTG TAC
       T  F  P  D  T  A  V  H  E  C  D  P  E  L  Y               540

1621  CGC GAG CTG CTA TTG CTC GTT TAC AAC AGA TGT CTG GTT GCC ACG
       R  E  L  L  L  L  V  Y  N  R  C  L  V  A  T               555

1666  AGC GAA GAG CTG CCT AAT GTC CAA GCT CCG CCA AAA ACA CGC CAC
       S  E  E  L  P  N  V  Q  A  P  P  K  T  R  H               570
                                 EcoRI
1711  GGA GCA TTT ACG TCT CTG CTG ATC CGA ATT CTG CCC CTG ATA CCG
       G  A  F  T  S  L  L  I  R  I  L  P  L  I  P               585

1756  TTT GAC GAA TAC CAA TCA TGG CTG GAG AGA ACC CTC AGT CTT GCG
       F  D  E  Y  Q  S  W  L  E  R  T  L  S  L  A               600

1801  TTC CGC ACA GTC GGC GAC GAG CGC ACA TAC CTT CTG GAC CTG CTC
       F  R  T  V  G  D  E  R  T  Y  L  L  D  L  L               615

1846  TGG GAC AGT ATT CTG GGC ACA AAC AGA CAC TAT CCG CAG AAA GGG
       W  D  S  I  L  G  T  N  R  H  Y  P  Q  K  G               630
                                    HincII
1891  TAC GTT GGC ATC CAA TGG TGG TAC GAA CAT GTC AAC GAG AGT CAG
       Y  V  G  I  Q  W  W  Y  E  H  V  N  E  S  Q               645

1936  GAA AAA GCA AAA TTA TAG TATTTAATCTACTCAGTTGGTTCCTGTTGGTCGAA
       E  K  A  K  L  -                                         650

1989  GAAATCAGCACAGCGCAGTTTGGATGTAGGATGTAACCAGGTTGGCTTTTCTTTCGGCT

2048  TCTTTCTTGCGCTGCCACTCCTCCAATCCAATGGTGCGTCGTTCGA
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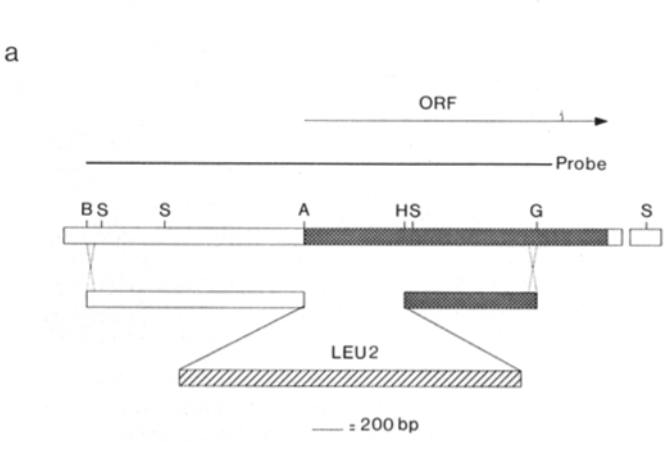
Figure 2. Nucleotide sequence and deduced primary sequence of the *PERI* gene. Some relevant restriction sites used for the different constructions are indicated above the nucleotide sequence. The initial complementing 3-kb fragment ended at the *Sau3A* site at position 1607. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z30206.



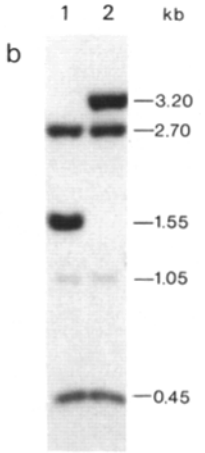


Figure 3. Disruption of the *PERI* gene. (*a*) The *LEU2* gene of *C. albicans* was inserted between the *Asp*718 (*A*) and the *Hin*dIII (*H*) site of the coding region of *PERI*. Subsequently, the obtained construct was introduced into the genome by homologous integration. (*b*) The correct integration was confirmed by Southern blotting of genomic DNA cut with *Sac*I (*S*) and probed with a *Bam*H1 (*B*)-*Bgl*1 (*G*) DNA fragment as indicated. Lane *1*, genomic DNA isolated from WT *H. polymorpha*; lane 2, genomic DNA isolated from the *perI::LEU2* disruption mutant.

variant of the PTS1 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 (PER1p) shows strong similarity to the consensus sequence $R1x_5H/QL$ 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 PER1p 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 PER1p (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 (*per1::LEU2*) indicated a correctly targeted chromosomal integration (Fig. 3 *B*). After mating of the *per1::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 *per1::LEU2* strain with the original mutant *per1-124/2D*, 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 *per1::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 PER1p 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 *per1::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., 1993*b*; Waterham et

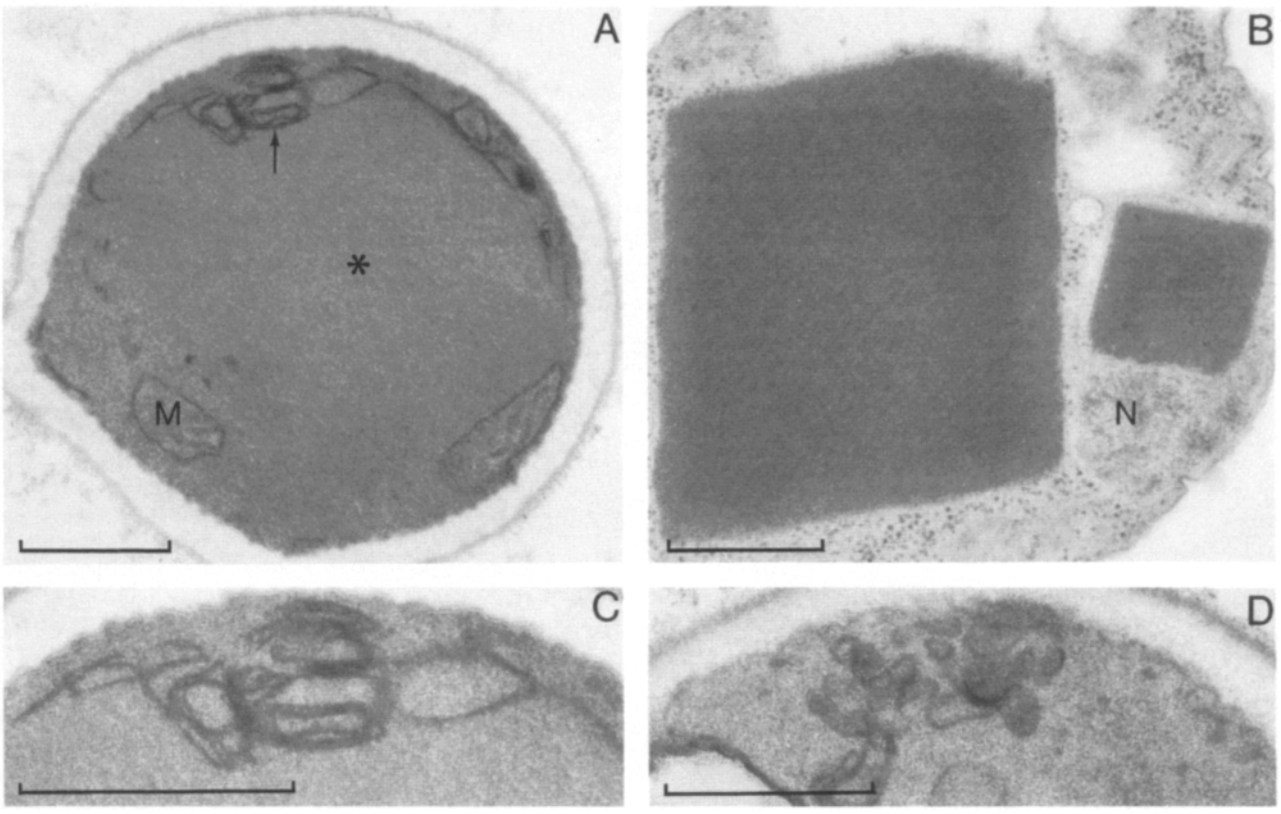


Figure 4. Morphology of the *per1::LEU2* disruption mutant grown in a continuous culture on a glucose/methanol mixture (*A–C*) or incubated on methanol in batch culture (*D*). The mutant displays large cytosolic crystalloids (*A*, *), composed of alcohol oxidase protein (*B*, glutaraldehyde/OsO_4). In these cells several vesicular structures were observed (*A*, *C*, and *D*, $KMnO_4$) which might represent peroxisomal remnants. *N*, nucleus. Bar, 0.5 μm.

al., 1993). Like all *H. polymorpha* Per⁻ mutants studied so far (Sulter et al., 1990, 1991), the *perl::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 PER1p is essential for peroxisome biogenesis in general.

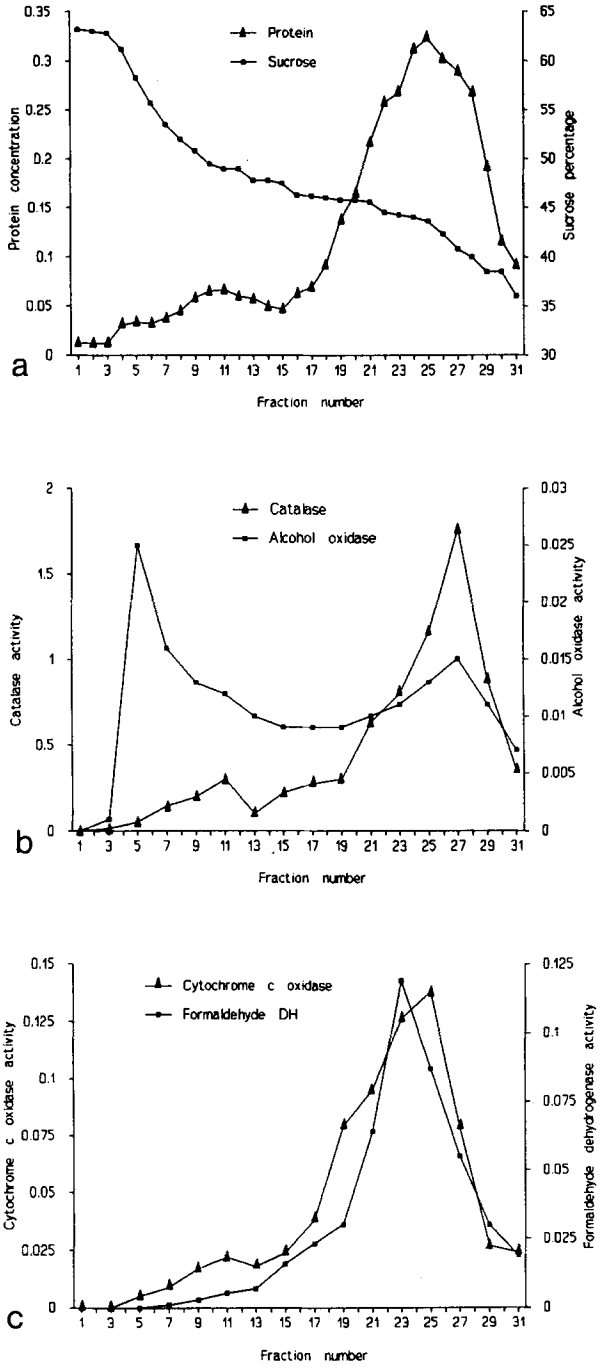


Figure 5. Subcellular fractionation of the *perl::LEU2* disruption mutant grown in a glucose/methanol limited continuous culture. The 30,000-*g* pellet obtained after differential centrifugation of homogenized protoplasts was fractionated on a sucrose gradient and divided in different fractions (see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

In order to further analyze the nature of the vesicular structures cells of the *perl::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 PER1 Gene

To obtain insight in the significance of PER1p 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

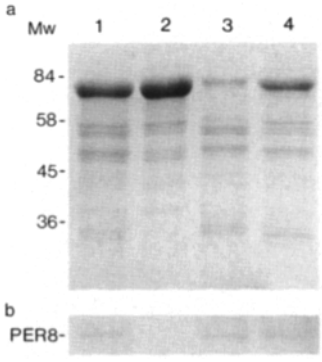


Figure 6. SDS-PAGE electrophoresis and Western blot analysis of fraction 11 obtained by cell fractionation of the *perl::LEU2* disruption mutant (Fig. 5), compared to purified peroxisomes of methanol-grown WT *H. polymorpha*. Proteins were separated on a 12.5% polyacrylamide gel and visualized by Coomassie 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.

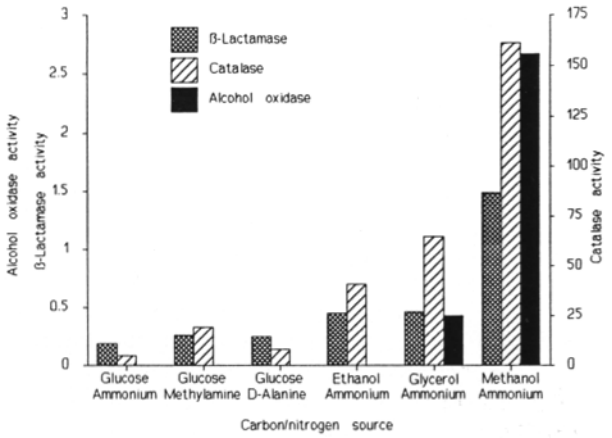


Figure 7. Expression of the *PER1* gene at various growth conditions. In order to establish *PER1* expression, the β-lactamase gene was cloned behind the promoter of *PER1* and transformed to *H. polymorpha*. The β-lactamase activity of variously-grown cells is given, together with the activities of catalase and alcohol oxidase as an indication for the rate of peroxisome development. β-lactamase activity was not detectable in untransformed WT cells. Enzyme measurements were performed on crude extracts prepared from cells grown in batch cultures and harvested at OD_{663} = 2 as described in Materials and Methods.

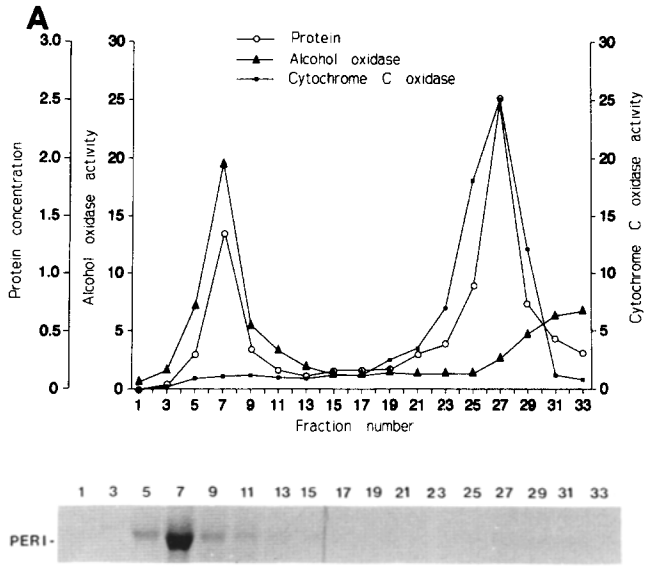


Figure 8. Subcellular localization of Perlp. (*A*) Methanol-grown WT cells of *H. polymorpha* were fractionated by differential centrifugation and subsequent sucrose gradient centrifugation. Peroxisomal (fraction 8) and mitochondrial (fraction 27) peak fractions are indicated by their marker enzymes, alcohol oxidase and cytochrome *c* oxidase, respectively. Protein concentrations are expressed as mg/ml, alcohol oxidase and cytochrome *c* oxidase as U/ml. (*B*) 20 μl of each odd fraction was run on a 7.5% SDS-PAA gel and blotted onto nitrocellulose for Western analysis using specific antibodies against Perlp.

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 methylamine, using the *PER1* 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 P_{PER1}-driven synthesis of bacterial β-lactamase, thus taking advantage of the sensitive β-lactamase activity assay. The results shown in Fig. 7 indicate that P_{PER1} 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 methylamine as nitrogen source. P_{PER1} 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 P_{PER1} 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 P_{MOX}. In glucose- or ethanol-grown cells no β-lactamase activity was detected, which indicates that carbon catabolite repression of P_{MOX} (Veenhuis and Harder, 1991) operates efficiently, even in the case of replicating plasmids. In methanol-grown transformants the P_{PER1} driven β-lactamase activity (1.5 units) is low compared to the activity resulting from expression behind P_{MOX} (100.4 units), which indicates that PER1p is a protein of low abundance in *H. polymorpha*.

The PER1 Gene Product Is a Peroxisomal Matrix Protein

Polyclonal antibodies raised against PER1p 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 PER1p observed after cleavage with factor Xa of the maltose-binding protein-PER1p fusion protein synthesized in *E. coli* (not shown). In crude extracts prepared of methanol-grown cells of WT *H. polymorpha* PER1p could hardly be detected, again indicating that PER1p is a low abundant protein. However, when these cells were fractionated by differential centrifugation and subsequent sucrose density centrifugation, PER1p was specifically detected in highly purified peroxisomes (Fig. 8 and 9). The occasionally costaining 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, PER1p was exclusively detected in the soluble fraction, indicating that PER1p is a component of the peroxisomal matrix (Fig. 8). As expected, PER1p 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 PER1p in WT *H. polymorpha* was also studied by immunocytochemistry, performed on thin sections of transformants in which the *PER1* gene was expressed behind the P_{MOX}. In these cells labeling was predominantly present at edge of the peroxisomes (Fig. 1 *D*). We have interpreted our combined data in that Perlp

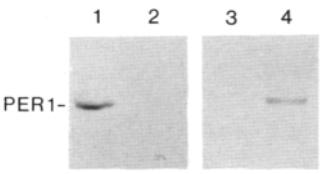


Figure 9. Localization of PER1p. Equal amounts of purified peroxisomes (lane *1*) and mitochondria (lane *2*) obtained after differential and subsequent sucrose density centrifugation of methanol-grown WT cells of *H. polymorpha* (compare Fig. 8, fraction 7 and fraction 28, respectively) were fractionated on a 7.5% SDS–polyacrylamide gel and blotted for Western analysis using specific antibodies raised against PER1p (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 μgr 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, PER1p contains both the carboxy-terminal PTS1 (AKL) and the amino-terminal PTS2 (KLx_5QL) 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 *per1::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., 1992*b*), 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 *per1* allele, thus giving rise to a WT *PER1* gene

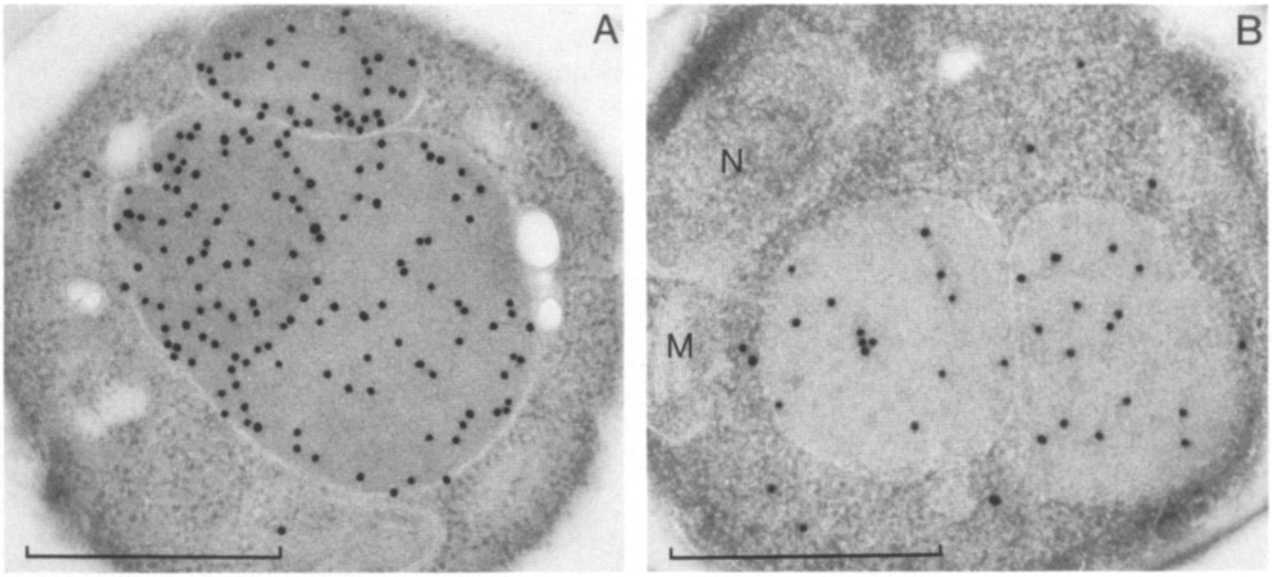


Figure 10. Peroxisomal targeting of bacterial β-lactamase protein by the 9 carboxy-terminal (*A*) and the 16 amino-terminal amino acids (*B*) of PER1p, as revealed by immunocytochemistry using specific antibodies against β-lactamase. *H. polymorpha* cells were transformed with the constructs detailed in Table II; transformants were grown in methanol-containing batch cultures. Bar, 0.5 μm.

in the genome. Several observations, however, indicated that this was not the case. First, after introduction of the incomplete gene on the pHRP2 vector in the original *perl-124/2D* mutant, all leucine prototrophic strains were complemented for growth on methanol. Second, the pHRP2 vector was readily recovered from the complemented mutant yeast strain by electrotransformation 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 PER1p 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, PER1p 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 PER1p 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 PER1p was localized in these organelles (H. R. Waterham and G. J. Swaving, unpublished results).

The biochemical studies showed that PER1p is a protein of low abundance located in the peroxisomal matrix. At present, PER1p 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 (M. Veenhuis, unpublished results). However, for *S. cerevisiae* a gene essential for peroxisome biogenesis is reported which contains a PTS1 signal and therefore might encode a peroxisomal matrix protein (PAS6p, Erdmann and Kunau, 1992).

Although we could not deduce a specific function for PER1p, we speculate that PER1p may play a role in triggering the protein import competence of individual peroxisomes. In earlier reports, we have shown that yeast peroxisomes are only temporarily capable to import newly synthesized matrix proteins. This import competence is related to the developmental stage of the organelles: newly developed peroxisomes are able to incorporate proteins whereas the large, mature organelles have lost this ability (Veenhuis et al., 1989; Waterham et al., 1992*a*). Our hypothesis for the role of PER1p is based on the presence of peroxisomal vesicular structures which lacked matrix proteins in the *perl::LEU2* disruption mutant. The presence of these vesicles has not been observed before in other *H. polymorpha per* mutants; in these mutants peroxisomal membrane proteins are primarily located in small protein-phospholipid aggregates (Sulter et al., 1993*b*; Waterham et al., 1993). Thus the induction of peroxisomal vesicles in the cells of the *perl::LEU2* disruption mutant suggests that protein import is blocked but that peroxisome multiplication (proliferation) is not affected. Therefore, the import of PER1p into preperoxisomal structures appears to be a prerequisite to import of other matrix proteins. The presence of two functional PTS sequences on PER1p suggests that a correct peroxisomal localization of the protein is critical for peroxisome development.

The proposed function for PER1p requires that PER1p is constitutively present. This is indeed the case, as was indicated by the expression studies which showed that PER1p is synthesized even in glucose-grown cells, where peroxisome induction is fully repressed. This is consistent with the fact that the small peroxisomes present in repressed WT cells are capable of importing peroxisomal matrix proteins (e.g., alcohol oxidase) synthesized during non-methylotrophic growth conditions (Distel et al., 1988). In view of the above hypothesis of the role of PER1p, this import would not occur if the protein was absent under these conditions. However, PER1p is not predicted to be a core protein of the peroxisomal protein import machinery since its role requires that the protein itself has to be imported into the organelles. The results of the targeting experiments indicate that this import most probably occurs via the same import routes as for other matrix proteins.

The putative role of PER1p may be linked with a physical interaction of the protein with the PER8 protein as was suggested from a classical genetic study, performed with 12 different *H. polymorpha PER* genes (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 P_{MOX} 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 PER1p and PER8p may coordinately regulate the development of new protein import competent peroxisomes by fission from preexisting organelles.

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