Pmp27 Promotes Peroxisomal Proliferation

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Abstract. Peroxisomes perform many essential functions in eukaryotic cells. The weight of evidence indicates that these organelles divide by budding from preexisting peroxisomes. This process is not understood at the molecular level. Peroxisomal proliferation can be induced in Saccharomyces cerevisiae by oleate. This growth substrate is metabolized by peroxisomal enzymes. We have identified a protein, Pmp27, that promotes peroxisomal proliferation. This protein, previously termed Pmp24, was purified from peroxisomal membranes, and the corresponding gene, PMP27, was isolated and sequenced. Pmp27 shares sequence similarity with the Pmp30 family in Candida boidinii. Pmp27 is a hydrophobic peroxisomal membrane protein but it can be extracted by high pH, suggesting that it does not fully span the bilayer. Its expression is regulated by oleate. The function of Pmp27 was probed by observing the phenotype of strains in which the protein was eliminated by gene disruption or overproduced by expression from a multicopy plasmid. The strain containing the disruption (3B) was able to grow on all carbon sources tested, including oleate, although growth on oleate, glycerol, and acetate was slower than wild type. Strain 3B contained peroxisomes with all of the enzymes of β-oxidation. However, in addition to the presence of a few modestly sized peroxisomes seen in a typical thin section of a cell growing on oleate-containing medium, cells of strain 3B also contained one or two very large peroxisomes. In contrast, cells in a strain in which Pmp27 was overexpressed contained an increased number of normal-sized peroxisomes. We suggest that Pmp27 promotes peroxisomal proliferation by participating in peroxisomal elongation or fission.

As cells divide, mechanisms exist to ensure that daughter cells receive their proper complement of organelles. In some cases, organellar division and segregation are tightly linked. The division of the nucleus directly preceding cytokinesis is perhaps the best known example. Some organelles can undergo proliferation independent of cell division. One example is the expansion of the endoplasmic reticulum that occurs in the developmental pathway of exocrine cells, or as a response to certain drugs (Michaopoulos et al., 1976), or to the overexpression of resident proteins (Jingami et al., 1987).

The proliferation of peroxisomes can also occur independent of cell division. Various agents can stimulate this process. In animal cells, proliferation can be caused by hypolipidemic drugs and plasticizers (Lock et al., 1989). In eukaryotic microorganisms, various natural growth substrates induce peroxisomal proliferation; peroxisomal enzymes are required to metabolize and assimilate these substrates to permit growth of the culture (Veenhuis and Harder, 1987). Systems of peroxisomal proliferation should be useful to understand the normal pathway of organelar assembly. Protein import accompanies peroxisomal proliferation. There has been much progress in the past several years in characterizing the import of matrix proteins (Subramani, 1993). Most proteins destined for this compartment contain the tripeptide sequence SKL, or a variant, at their extreme carboxy termini (Gould et al., 1989). This motif is known as peroxisomal-targeting sequence 1 (PTS1). A few peroxisomal matrix proteins, notably 3-ketoacyl thiolase, contain a different motif, termed PTS2, at their amino termini. Both PTS1 and PTS2 are sufficient for import of carrier proteins from the cytosol (Gould et al., 1989; Swinkels et al., 1991). A candidate for the PTS1 receptor has been identified in yeast (McCollum et al., 1993). Import requires ATP (Imanaka et al., 1987; Wendland and Subramani, 1993) and may require a proton gradient (Bellion and Goodman, 1987). Recent data indicate that oligomeric proteins are competent for peroxisomal import (Glover et al., 1994; McNew and Goodman, 1994), suggesting that assembly of cytosolic precursors into peroxisomes may be fundamentally different from import into other organelles.

1. Abbreviations used in this paper: AEBSF, 4-(2-aminoethyl)-benzylsulfonfylfluoride; PTS, peroxisomal-targeting sequence.
Several genes have recently been isolated in yeast that are required for peroxisomal assembly (Subramani, 1993). Many of these are assumed to participate in protein import, since in their absence, matrix proteins remain in the cytoplasm, or are degraded there. Homologous gene products in humans probably exist, and mutations in these genes may be responsible for some of the peroxisomal diseases, several of which lead to death at an early age (Lazarow and Moser, 1989).

In contrast to protein import into peroxisomes, the mechanism of peroxisomal proliferation per se is poorly understood. Peroxisomes can be formed by budding or fission from preexisting peroxisomes (Lazarow et al., 1980), and peroxisomal matrix and membrane proteins are assembled from the cytosol. We have used the methylotrophic yeast, *Saccharomyces cerevisiae*, as a model system to dissect the steps of peroxisomal assembly. The procedure of McNew et al. (1994) was used to obtain 25,000 g organellar pellets from a 500-ml culture of oleate-grown cells was resuspended in 200 μl of 1 M sorbitol, 5 mM MES-NaOH, pH 5.5, and 0.2 mM 4-(2-aminoethyl)-benzylsulfonylfluoride (AEBSF). 50 μl of the suspension was subjected to extraction with Triton X-114 according to the method of Bordier (1981) as modified by Brusca and Radolf (1994). The detergent was removed from the aqueous phase by a second extraction with chloroform:methanol (2:1) and then water was added to the final organic phase. The water phase was then removed by aspiration. Membrane lipids are presumed to be provided by the endoplasmic reticulum, although lipid bodies support this function in plants (Chapman and Trelease, 1991). Both peroxisomal matrix and membrane proteins are assembled from the cytosol. We have used the methylotrophic yeast, *Candida boidinii* as a model system to dissect the steps of peroxisomal proliferation (Veenhuis and Goodman, 1990), since this process occurs rapidly and massively in this species in response to methanol (Sahm et al., 1975). Without methanol, a few small peroxisomes exist in the cell. Upon addition of this carbon source, there is an initial expansion and elongation of the peroxisomal membrane in a subset of these organelles. This is accompanied by the expression of at least one peroxisomal membrane protein, Pmp47. Peroxisomes then undergo fission, creating clusters of small organelles. Finally, the organelles gain volume as the matrix proteins are massively induced and imported.

We now report that Pmp27, a peroxisomal membrane protein from *Saccharomyces cerevisiae*, promotes peroxisomal proliferation. This protein, previously termed Pmp24, was identified as an abundant constituent of the peroxisomal membrane in this yeast (McCammon et al., 1990). The phenotypes of strains in which Pmp27 is not expressed or overexpressed are consistent with the hypothesis that this protein participates in peroxisomal elongation and fission.

### Materials and Methods

#### Strains and Culture Conditions

**MMYO11** (McCammon et al., 1990), transformed with plasmids when appropriate, was used for all experiments except where noted. **MMYO30**, a derivative of W303-la (U.C. Berkeley Yeast Genetic Stock Center, Berkeley, CA) adapted for growth on oleic acid, was mated to **MMYO11** to generate **MMYODIPI**, the host strain for the Pmp27 disruption. The strains 3A-3D were tetrad products resulting from sporulation of **MMYODIPI** after the disruption. Strain 3B, containing the disrupted gene, was used as the recipient for further transformations as indicated (Table I).

For growth on oleate-containing medium, strains were routinely cultured in minimal medium (0.67% Yeast Nitrogen Base [Difco Labs, Detroit, MI] and carbon source) containing the appropriate amino acids and base supplements to complement auxotrophic markers and to prevent loss of the plasmids. For typical experiments in which organelles from cells were fractionated, peroxisomes were induced to proliferate by the method of McNew and Goodman (1994). Cells were harvested 18–24 h after addition of oleate. For the experiment in which organelles were isolated from the tetrad strains (see Fig. 8), cells were grown overnight in YPD, and then back diluted into YPGOT (containing 3% glycerol, 0.1% oleate and 0.25% Tween 40; Zhang et al., 1993) at an OD<sub>600</sub> of 0.5 and grown once more overnight before harvesting.

For the comparison of growth of the four tetrad strains (3A-3D) on plates other than those with oleate, cells were replica-plated from a YPD plate onto plates which contained 2% yeast extract, 1% peptone, and one of the following carbon sources: 2% potassium acetate, 3% glycerol, 2% sucrose, 2% raffinose, 3% ethanol, or 2% galactose. For growth on oleate plates, cells were streaked from YPD onto HMYO medium (McCammon et al., 1990) containing 0.25% Tween 40.

#### Organellar Fractionation and Examinations

The procedure of McNew et al. (1994) was used to obtain 25,000 g organellar pellets and to fractionate organelles on Nycodenz gradients. For organellar isolation from the four tetrads (see Fig. 8), Zymolyase 20T at 2 mg/l,000 OD<sub>450</sub> units of cells was used instead of Zymolyase 100T to generate yeast spheroplasts.

For the Triton X-114 (Sigma Chem Co., St. Louis, MO) extractions, a 25,000 g organellar pellet from a 500-ml culture of oleate-grown cells was resuspended in 200 μl of 1 M sorbitol, 5 mM MES·NaOH, pH 5.5, and 0.2 mM 4-(2-aminoethyl)-benzylsulfonylfluoride (AEBSF). 50 μl of the suspension was subjected to extraction with Triton X-114 according to the method of Bordier (1981) as modified by Brusca and Radolf (1994). The proteins from the detergent phase were precipitated with 10 vol of ace-
tone and the proteins from the aqueous phase were precipitated with 10% TCA (final volume) before SDS-PAGE and immunoblotting.

For extractions with sodium carbonate, a 25,000 g organellar pellet from oleate-grown cells was resuspended in 1 M sorbitol, 5 mM MES-NaOH, pH 5.5, and 0.2 mM AEBBS and was diluted 20-fold in ice cold 100 mM NaCl, 50 mM Na2CO3 (Fujiki et al., 1982). After 1 h, the sample was centrifuged at 195,000 g for 60 min. The supernatant was removed, neutralized with 15 μl of concentrated HCl, and then precipitated with 10% TCA (final volume). Samples were analyzed by SDS-PAGE and immunoblotting.

**Cloning of the PMP27 Gene**

Peroxisomal membranes were isolated from oleate-grown cells (McCann et al., 1990) and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose and the 27-kD region was subjected to trypsin digestion. Pmp27 tryptic fragments were sequenced as described (Moreno et al., 1994). To isolate Pmp27, two degenerate oligonucleotides were synthesized based on the ends of the amino terminal amino acid sequence. They were used to amplify a PMP27 fragment from genomic DNA. Several closely related PCR products were obtained, and two new nonoverlapping oligonucleotides were generated based on a consensus of the PCR products: 5'GTGTCGCTGACATCTTGGTTTTA(T/G)CACCACATGTTT3' and 5'GTC- AAGTCTT(C/T)CAGTTGCTT(G/C)CTTGCTCAGGCTTGAAGAGT-3'. These two oligonucleotides were radiolabeled and used to probe a YCP50-based genomic library (Rose et al., 1987). One clone, M1, was identified that hybridized to both probes. A 3.9-kbp HindIII-SphI fragment (see Fig. 6 A) from M1, which also hybridized to both probes, was subcloned into pUC19, generating plasmid NT. This fragment contained the Prop27 coding sequence. This region was replaced by the URA3 gene derived from one typical tetrad (3A-3D) were analyzed in detail, as described in Results.

**Expression of Pmp27 from Plasmids**

For pRS27, the BamHI-ClaI fragment of N7, which contained the open reading frame and 650 bases upstream from the start site, was ligated into pRS426. For pRS313, the BamHI-ClaI fragment of N7, which contained the open reading frame, from base 55 to 367 (see Fig. 1), was replaced by URA3. PMP27KO was digested with NgoMI, SauI, and AatII (digestion of the vector sequence with AatII was not complete). These two oligonucleotides were radiolabeled and used to probe a YCp50-based genomic library (Rose et al., 1987). One clone, M1, was identified that hybridized to both probes. A 3.9-kbp HindIII-SphI fragment (see Fig. 6 A) from M1, which also hybridized to both probes, was subcloned into pUC19, generating plasmid N7. This fragment contained PMP27. The coding region was completely sequenced in both directions using Sequenase (United States Biochem. Corp., Cleveland, OH).

**Integrative Disruption of Pmp27**

An internal region of the PMP27 open reading frame, from XbaI to Agel, was replaced with the URA3 gene (see Fig. 6 A). The plasmid pKM (gift of Karsten Melcher, U.T. Southwestern Medical Center, Dallas) consisting of URA3 on a HindIII fragment cloned into pUC19, was used for this purpose. pKM was digested with XbaI and XmaI, and the URA3-containing fragment was isolated. Meanwhile, the BgHI-BamHI fragment containing PMP27 was isolated from N7 and subcloned into pUC19. The resultant 2.9-kbp fragment was used for transformation of MMYODIP1 as the high-copy plasmid negative control. All four plasmids were singly transformed into strain 3B and expression of Prop27 was checked by immunoblotting as described in Results.

**Antibodies to Pmp27**

The predicted protein sequence of Pmp27 was analyzed by the antigenic index algorithm of Jameson and Wolf (1988). The most antigenic portion of the protein, amino acids 169-181, plus an additional cysteine for coupling (NH2-CGDEHEDHKVLSG-COOH), was synthesized (Clive Slaughter, UT Southwestern, Dallas, TX). This peptide was coupled to keyhole limpet hemocyanin using n-naleimidobenzyl-n-hydroxysulfosuccinimide ester (Pierce, Rockford, IL), and the product was injected subcutaneously into two New Zealand white rabbits. Antibodies from rabbit J786 were used for immunoblotting. Before use, the antibodies were affinity purified by binding to a peptide-Sepharose column and eluting with 0.2 M glycine, pH 2.2.

**SDS-PAGE and Immunoblotting**

Standard 9% Laemmli gels (Laemmli, 1970), with the separating gel at pH 9.2, were employed. Immunoblotting was performed as described by Tow- bin et al. (1979) using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) for detection. The thiolase antibody for the immunoblot was kindly provided by Jon Rothblatt (Dartmouth University, Hanover, NH). Antibodies against acyl CoA oxidase and multifunctional enzyme were generated from denatured antigens as described previously (McNew et al., 1993). Primary antibodies were used at the following concentrations for immunoblots: anti-Pmp27 (affinity purified) 1:1,000; anti-acyl CoA oxidase (IgG fraction) 1:500; anti-multifunctional enzyme (IgG) 1:500; anti-thiolase 1:40,000; anti-porin (gift of G. Schatz, Biocenter, Basel) 1:1,000. Molecular weight standards were from Bio-Rad Labs (Hercules, CA). Quantiﬁcation of X-ray ﬁlms was performed on a Zenith Soft Laser Scanning Densitometer.

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**Figure 1. Sequence of PMP27.** The inferred amino acid sequence of Pmp27 is over the nucleotide sequence. The amino terminal and internal tryptic peptide sequences are boxed. The arrows are drawn over the probable olate response element. A possible TATA box and polyadenylation signal are underlined in the 5' and 3'-untranslated regions, respectively. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z46846.
Figure 2. Pmp31 and Pmp27 are related sequences. Kyte-Doolittle hydropathy plots (Kyte and Doolittle, 1982) of Pmp31 from 
*Candida boidinii* and Pmp27 from *S. cerevisiae* are shown. The window of analysis is 19 amino acids. The dashed lines indicate the thresh-
old for theoretical membrane-spanning domains. I, II, and III are hydrophobic patches as described in the text. Thick horizontal lines 
underneath the tracings indicate regions of sequence similarity. Numbers show % identity (% similarity).

**RNA Isolation and Northern Blotting**

RNA was isolated according to the protocol of Köhrer and Domdey (1991). 
10 \( \mu \)g of total RNA was subjected to electrophoresis in glyoxal agarose gels 
(Thomas, 1980). The RNA was transferred to nylon (Zeta- probe, Bio-Rad Labs) for 2 d in 25 mM sodium phosphate, pH 6.8. Prehybridization 
and hybridization were performed in Church-Gilbert solution (Church and Gil-
bert, 1984) at 55°C. Blots were probed with the PstI fragment of *PMP27*, 
which was radiolabeled after random priming (Random Primed Labeling 
Kit, Boehringer Mannheim). The blot was subsequently stripped in 0.1x 
SSC and 0.5% SDS at 95°C for 1 h. A fragment from the yeast actin gene 
was similarly radiolabeled and used as a control for loading. Quantitation 
of Northern blots was performed using radioanalytic imaging (AMBIS, San 
Diego, CA).

**Electron Microscopy**

Whole cells were fixed in 1.5% (wt/vol) KMnO₄ for 20 min at room tem-
perature. 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 cacodylate buffer (pH 7.2) for 90 min at 0°C, dehydrated in a 
graded ethanol series and embedded in Unicryl. Immunolabeling was per-
formed on ultrathin sections with specific polyclonal antibodies against 
thiolase (gift of Wolf Kunau, University of Böchum, Germany) and gold-

Figure 3. Pmp27 is a perox-
isomal protein. (S and P) Su-
pernatant and pellet resulting 
from a 25,000-g centrifuga-
tion; 0.5 % of each was loaded 
on gels. Asterisks denote mo-
lecular mass standards. Num-
bers signify fractions from the 
Nycodenz gradients, from 
bottom to top; 20 μl of each 
fraction were loaded. (A) 
Coomassie-stained gel. Mo-
lecular masses of the stan-
dards are 200, 116, 97, 66, 45, 
31, 21.5, and 14.4 kD. (B) Im-
munoblot from a gel identical 
to the one in A. Acyl CoA oxi-
dase and thiolase were blotted 
together. Horizontal lines to 
the right of the blot denote 
standards as follows: the porin 
immunoblot: 35.1 and 297 
kD; the acyl CoA oxidase and 
thiolase immunoblot: 142.9, 
97.2, 50, and 35.1 kD; the 
Pmp27 immunoblot: 29.7 and 
21.9 kD.
Results

Pmp27 Is Similar to the Pmp30 Family from Candida boidini

to isolate the gene encoding Pmp27, we first cultured cells in oleate to induce peroxisomal proliferation (McCammon et al., 1990), and then purified the protein from peroxisomal membranes by SDS-PAGE. We obtained partial protein sequence which was used to generate oligonucleotide probes to screen a S. cerevisiae genomic library. One clone was obtained from the screen that contained an open reading frame predicting a protein of 237 amino acids, or 26.9 kD (Fig. 1). The predicted mass was within the range that we observed for Pmp27 on protein gels, and it contained the two partial sequences which we had identified from the isolated protein. With knowledge of the inferred mass of the protein, we have decided to change the name of the protein from Prop24 to Pmp27. A putative oleate response element, which is found in other peroxisomal proteins in S. cerevisiae (Einerhand et al., 1993), preceded the open reading frame. These data indicated that we had cloned PMP27.

A search of GenBank (release 82.0) revealed that Pmp27 shares extensive sequence similarity with the Pmp30 family from Candida boidini. This family consists of Pmp31 and Pmp32 from the polyploid strain ATCC No. 32195 (American Type Culture Collection, Rockville, MD) (Goodman et al., 1986; Moreno et al., 1994) and Pmp30 from the haploid strain AO1-1 (Sakai, Y., and J. M. Goodman, manuscript in preparation). These proteins are abundant in peroxisomal membranes of C. boidini. They are induced by the three growth substrates methanol, oleate, and d-alanine. Since these compounds cause the expression of different constellations of peroxisomal matrix proteins (Goodman et al., 1990), we had previously postulated that members of the Pmp30 family provide a function related to peroxisomal assembly or structure, rather than play a specific metabolic role.

The similarity of Pmp27 and Pmp31 (as an example of this family) is shown in the context of hydrophathy plots (Fig. 2). Regions of sequence similarity are indicated by the thick horizontal bars beneath the tracings. Taken together, common elements of structure emerge. The amino termini of these two proteins appear amphipathic. The first 40 amino acids comprise the most conserved part of these proteins (69% identity). This region is followed by two patches of greater hydrophobicity, denoted in the figure as I and II. Patch I is particularly hydrophobic in Pmp27. Patch II is followed by the most hydrophilic, and least conserved, part of the proteins. Finally, the protein terminates in another hydrophobic region, patch III. Patch III is more hydrophobic in Pmp31 than in Pmp27. These data indicate that the proteins have hydrophobic character but no good candidate for a membrane-spanning domain. The conservation between the two sequences in all but the most hydrophilic regions suggests that the role of these proteins, assuming that they are functional homologs, is related to their interaction with the membrane.

Pmp27 Is Tightly Bound to Peroxisomal Membranes but Is Probably Not an Integral Membrane Protein

Although we have shown previously that Pmp27 is a compo-
Peroxisomal Location of Pmp27

Similar to the response in mRNA levels to oleate, a dramatic rise in Pmp27 was also seen, reaching peak concentrations ~6 h after addition of the carbon source. Kinetics of induction of this protein were similar to that of two enzymes of peroxisomal β-oxidation, acyl CoA oxidase, and thiolase. This is in contrast to the induction of the C. boidinii protein Pmp47, which precedes induction of the matrix proteins (Veenhuis and Goodman, 1990).

PMP27 Is Required for Optimal Growth in Glycerol, Acetate, and Oleate Media

The similarity of the sequence of Pmp27 with members of the Pmp30 family, and the vigorous induction of Pmp27 by oleate, suggested that Pmp27 was important for peroxisomal function. To gain more information in this regard, we decided to study peroxisomes in strains in which PMP27 was disrupted or in which Pmp27 was overexpressed. To construct the disruption strain, we replaced 44% of the PMP27 coding sequence with the auxotrophic marker URA3 (Fig. 6 A). The disruption was performed in a diploid in case the gene had been essential for viability. To obtain haploid disruptants, uracil prototrophs were subjected to sporulation. An analysis of genomic DNA and growth on selective medium of 3 tetrads confirmed that uracil prototrophy and the gene disruption cosegregated. A Southern blot with one tetrad, 3A-3D, is shown in Fig. 6 B. The analysis shows that strains 3A and 3B, which were uracil prototrophs, contained the gene disruption, while strains 3C and 3D had the intact gene. No other PMP27-like genes were detected in this analysis.

To begin to analyze the disruption strain, we compared growth of strains 3A-3D on various carbon sources. The strains all grew on plates which had galactose, glucose, acetate, glycerol, sucrose, rafinose, or ethanol as the sole carbon source at both 30°C and at 37°C. They also grew on oleate plates at both temperatures, suggesting that functional peroxisomes were present (data not shown). In contrast, petite mutants (the negative control) failed to grow on the oleate plates. This was expected since mitochondrial function is required for use of this carbon source.

We examined the rate of growth in liquid medium with selected carbon sources to determine whether the absence of Pmp27 produced any differences in the rate of growth (Fig. 7). For these experiments, we used the disruption strain 3B which was transformed with a low copy CEN-based plasmid in which PMP27, driven by its own promoter, was either present or absent (strains 3B[pRS27] and 3B[pRS313], respectively). The absence of Pmp27 had no effect on growth in glucose. The gene had a small but reproducible effect on acetate. In contrast, the disruption strain grew much worse on glycerol and oleate medium. Our control strains only double once or twice in oleate liquid medium. In the absence of oleate, the OD of neither strain increased. The effect on glycerol growth might reflect an abnormal interaction between the peroxisomal glyoxylate cycle and the mitochondrial Krebs cycles (see Discussion).

PMP27 Is Not Required for the Import of Peroxisomal Matrix Proteins

To test whether the PMP27-disruptant strains contained peroxisomes, organelles and postorganellar supernatants in the strains 3A-3D were prepared as usual and analyzed by immunoblotting (Fig. 8). It is clear that the three enzymes of β-oxidation (acyl CoA oxidase, multifunctional enzyme, and thiolase) are predominantly in the organelar pellets in all four strains. In contrast, Pmp27 is only detected in strains...
Figure 6. Disruption of PMP27. (A) The disruption strategy is shown. The upper line denotes the genomic fragment in clone N7; the arrow indicates the PMP27 coding sequence. Agel and Xmal are compatible sites. Details are described in Materials and Methods. (B) Southern blot of Tetrad 3 and the parental strain. Genomic DNA was restricted with PstI. The blot was probed with the PMP27 PstI fragment. The wild-type gene yields a fragment of 500 bp while the disruption yields an 1,150-bp band. The weak hybridization to 3A and 3B presumably is caused by the absence of much of the corresponding gene.

Figure 7. The PMP27 disruption affects growth in glycerol, acetate, and oleate media. The disruption strain 3B was transformed with either pRS313 (dashed lines) or pRS27 (containing PMP27, solid lines) and cultured in the media as indicated. Growth was monitored by light scattering at 600 nm. For all cases except oleate medium, cells were precultured in the identical media. For oleate medium, cells were precultured as previously described (McNew and Goodman, 1994). Parallel controls in the absence of oleate showed no increase in OD after 48 h.
To determine whether the absence of Prop27 were first scrutinized. Strain 3C, containing peroxisomal morphology, we examined our strains by electron microscopy. Spore products of the tetrad (strains 0.1-0.3 μm diameter were seen in a typical cross-section after staining and immunoblotting with antibodies against acyl CoA oxidase and thiolase (not shown). Thus, Pmp27 does not affect the protein composition of peroxisomes at this level of detection.

**Pmp27 Promotes Peroxisomal Proliferation**

To determine whether the absence of PMP27 had any effect on peroxisomal morphology, we examined our strains by electron microscopy. Spore products of the tetrad (strains 3A-3D) were first scrutinized. Strain 3C, containing PMP27, was similar in appearance to the parental strain MMYO11 (McCammon et al., 1990) in that several peroxisomes of 0.1-0.3 μm diameter were seen in a typical cross-section after growth on oleate (Fig. 9 A). In contrast, one or two giant peroxisomes, 0.5-1.0 μm in diameter, as well as several normal-sized peroxisomes, were seen in the disruptant strain 3B (Fig. 9 B). Analysis with antibodies against thiolase confirmed that these organelles were indeed peroxisomes. It is interesting to note that a disruption of PMP30 in C. boidinii AOU-I also yielded larger than normal peroxisomes (Sakai, Y., and J. M. Goodman, manuscript in preparation).

To more carefully control the experiment, we repeated the analysis on strains 3B(pRS27) and 3B(pRS313). Pmp27 expressed from a CEN plasmid reverted strain 3B to having small peroxisomes similar in size and number to wild type (Fig. 9 C).

One interpretation of these results is that the absence of Pmp27 inhibited peroxisomal fission during proliferation, even though the organelles remained competent for protein import. To further test this hypothesis, we placed PMP27 on a multicopy plasmid to observe the effects of overproduction of the protein, and introduced the plasmid into strain 3B, creating strain 3B(YEplac112+PMP27). If Pmp27 promotes peroxisomal proliferation, then overexpression might lead to an overabundance of the organelle. This is what was seen (Fig. 9, D-F). Elongated clusters of proliferating peroxisomes are clearly observed after 6 h of oleate induction (Fig. 9 D). By 24 h, much of the cytoplasm was filled with peroxisomes (Fig. 9 F). Overproduction of the protein was verified by immunoblotting (data not shown). This effect on proliferation was not observed in the parallel plasmid control strain 3B(YEplac112), nor with any other of our control strains (data not shown). Our data indicate that Pmp27 promotes peroxisomal proliferation.

**Discussion**

We have previously reported that the most abundant peroxisomal membrane protein in Saccharomyces cerevisiae is Pmp27, originally termed Pmp24 (McCammon et al., 1990). Here we present its inferred primary sequence, biochemical characterization, and experiments that indicate a role in peroxisomal proliferation. Our hypothesis is that this protein promotes peroxisomal fission, perhaps at the step of organelle elongation.

Our data strongly suggest that Pmp27 is the structural and functional homolog of the Pmp30 family of C. boidinii. We have analyzed the expression of all of the abundant PMPs of C. boidinii and have categorized them into substrate-specific and substrate nonspecific groups. Pmp20 is induced only by methanol and we suspect it participates in the metabolism of that substrate. In contrast, Pmp31-32 and Pmp47 are induced by three diverse peroxisomal proliferators. For this reason we suspected that these proteins participate in biogenesis of the organelle (Goodman et al., 1990). This hypothesis is now extended to Pmp27 of S. cerevisiae. We are presently performing functional complementation experiments in both yeasts, in collaboration with Y. Sakai (University of Kyoto). The extractability of Pmp27 from a crude organelar pellet by sodium carbonate was surprising in light of its behavior in Triton X-114 and our past observation that Pmp27 was the major constituent of carbonate-extracted peroxisomal membranes. These data are reconciled by the observation that purification of peroxisomal membranes leads to greater inextractability of Pmp27 with this reagent. We believe that the
behavior of Pmp27 from the crude organellar pellet is more representative since there has been less opportunity for oxidation or denaturation. The reason for apparent extractibility of the protein cannot be caused by microvesiculation of the peroxisomal membrane such that it is no longer sedimentable, since ~50% of exogenously expressed Pmp47, an integral membrane protein, is recovered in the carbonate pellet in this experiment (Marshall, P. A., and J. M. Goodman, unpublished result). Our conclusion based on extraction and detergent solubility data is that Pmp27 is a peripheral membrane protein that strongly interacts with the bilayer. Topology studies will be required to determine which side of the membrane it faces.

We disrupted PMP27 to study its function. We observed that although strain 3B could grow on plates with all carbon sources tested, its growth was much less than that of the control strain in liquid medium containing glycerol or oleate. Metabolism of glycerol requires the shuttling of metabolites between the glyoxylate cycle in peroxisomes and the Krebs cycle in the mitochondria (Tolbert, 1981). For example, glycerol must use the gluconeogenic pathway via the glyoxylate cycle to produce serine and glycine, and the aspartate necessary for the glyoxylate cycle must be synthesized by the Krebs cycle (Melcher and Entian, 1992). Assuming that peroxisomes in cells grown in glycerol are larger without Pmp27, perhaps the decrease in peroxisomal surface area with respect to volume does not allow sufficient diffusion of metabolites in and out of peroxisomes, slowing the growth of cells on this carbon source. The lack of effect in dextrose medium confirms the minimal role of peroxisomal proliferation on this carbon source, since only tiny peroxisomes are seen in this growth condition (Veenhuis et al., 1987).
It is not known whether peroxisomal proliferation in S. cerevisiae follows the same temporal pattern that we have seen in C. boidinii. In this methylotroph, the induction of Pmp47 occurs before the abundant matrix proteins, suggesting that this PMP is important for an early event of proliferation (Veenhuis and Goodman, 1990). The coordinated induction of Pmp27 with the matrix proteins suggests that this protein acts at the same time as protein import. While fission and matrix growth can be temporally distinguished in C. boidinii, these processes may be simultaneous in S. cerevisiae. That is, peroxisomal budding occurs as the matrix expands. Such a pattern is seen in the yeast Hansenula polymorpha (Veenhuis et al., 1979) and in normal rat liver (Fahimi et al., 1993).

A striking effect of Pmp27 overproduction is the appearance of elongated peroxisomal structures at early times of proliferation. Such structures have been observed in C. boidinii in response to methanol (Veenhuis and Goodman, 1990). We suggest that these forms are necessary for efficient peroxisomal fission and that Pmp27 promotes their formation. Perhaps Pmp27 interacts with the cytoskeleton to cause organelle elongation. If, in contrast, Pmp27 is on the matrix side of the membrane, tubule formation would be possible if Pmp27 underwent homotypic or heterotypic polymerization. Thus, in the absence of Pmp27, fission does not readily occur, leading to large peroxisomes.

A point not addressed in this paper is whether Pmp27 is involved both in division of preexisting peroxisomes on repressing medium, or only in proliferation in response to peroxisomal inducers. A low but detectible concentration of Pmp27 is observed in glucose medium, suggesting a function in this carbon source. However, peroxisomes in the disruption strain can still undergo proliferation after many cell divisions in glucose medium. If division of basal peroxisomes were inefficient, one might expect a loss of organelles from the cells over time, leading to the inability of the strain to undergo proliferation. Since this was not observed, Pmp27 is probably not required for basal peroxisomal division.

Another peroxisomal protein, PER8 from Hansenula polymorpha, has very recently been shown to cause an increase in peroxisomal number when it is overexpressed (Tan et al., 1995). However, while deletion of PMP27 causes large peroxisomes, deletion of PER8 causes the disappearance of the organelle. Per8p is an integral membrane protein that contains cysteine-rich "zinc-finger"-like domains. It will be interesting to probe for functional interactions between Per8p and Pmp27. Additionally, another yeast gene, MMM1, has recently been described that affects mitochondrial shape (Burgess et al., 1994). In the absence of the corresponding protein, mitochondria are much larger than normal, leading to defects in the segregation of this organelle. We noted that the hydrophobic domain of Mmm1p (consisting of amino acids 82-122) is 26% identical in sequence to amino acids 67 to 107 to Pmp27. Perhaps similar functions of organelar fission are served by these two proteins.

We have found a peroxisomal protein that is important for peroxisomal proliferation, not for protein import. Could this protein be important in human peroxisomal diseases? The best studied diseases, such as Zellweger syndrome and neonatal adrenoleukodystrophy, have protein import defects. However, several lines of fibroblasts from patients with these diseases appear to import proteins at easily detectable levels (Gould, S., Johns Hopkins, personal communication). Moreover, peroxisomes appear larger in many of these lines. There are also two diseases that are less well characterized, pseudo-Zellweger (Huges et al., 1990) and pseudo-neonatal adrenoleukodystrophy (Poll-The et al., 1988). Cells from patients with these diseases have defects in peroxisomal fatty acid oxidation, but they also have large peroxisomes. Perhaps there is a role of a human Pmp27 homolog in these diseases.

We expect that Pmp27 has an important role in peroxisomal proliferation. In its absence, it is not difficult to imagine that aberrant peroxisomal fission could lead to slow exchange of metabolites between peroxisomes and the cytoplasm or other organelles, as well as to improper segregation of organelles during cell division.

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Note Added in Proof. In independent research, conclusions similar to our own have been recently reported in this journal (Erdmann, R., and G. Blobel. 1995. Giant peroxisomes in oleic acid-induced Saccharomyces cerevisiae lacking the peroxisomal membrane protein Pmp27p. J. Cell Biol. 128:509-523).

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