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.

A s 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 (Michalopoulos 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 organellar 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).[1] 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.

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1. *Abbreviations used in this paper:* AEBSF, 4-(2-aminoethyl)-benzylsulfonylfluoride; PTS, peroxisomal-targeting sequence.

Table I. Yeast Strains Used in This Study

Strain	Genotype	Reference
MMYO11	*MATα, ade-2-1, his3-11,15, leu2-3,112, trp1-1,ura3-1, can1-100*, Ole⁺	McCammon et al., 1990
MMYO30	*MATa, ade 2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*, Ole⁺	This study
MMYODIP1	*MATα/MATa, ade 2-1/ade2-1, his3-11,15/his3-11,15, leu2-3,112/leu2-3,112, trp1-1/trp1-1, ura3-1/ura3-1, can1-100/can1-100*, Ole⁺	This study
3A	*MATa, PMP27::URA3, ade 2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*, Ole⁺	This study
3B	*MATα, PMP27::URA3, ade 2-1, his3-11,15, leu2-3, 112, trp1-1, ura3-1, can1-100*, Ole⁺	This study
3C	*MATα, ade 2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*, Ole⁺,	This study
3D	*MATa, ade 2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*, Ole⁺,	This study
3B(pRS313)	*MATα, PMP27::URA3, ade 2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*, Ole⁺, (pRS313)	This study
3B(pRS27)	*MATα, PMP27::URA3, ade 2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*, Ole⁺, (pRS313+PMP27)	This study
3B(YEplac112)	*MATα, PMP27::URA3, ade 2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*, Ole⁺, (YEplac112)	This study
3B(YEplac112 + PMP27)	*MATα, PMP27::URA3, ade 2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*, Ole⁺, (YEplac112+PMP27)	This study

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 this is considered to be the normal route of peroxisomal division. 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 MMYODIP1, the host strain for the *PMP27* disruption. The strains 3A-3D were tetrad products resulting from sporulation of MMYODIP1 after the disruption. Strain 3B, containing the disrupted gene, was used as the recipient for further transformations as indicated (Table I).

Except 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_{600} 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 Extractions

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/1,000 OD_{600} 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 AEBSF and was diluted 20-fold in ice cold 100 mM Na$_2$CO$_3$ (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 (McCammon et al., 1990) and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose and the 27-kD region was subjected to tryptic 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'-GTCTG(T/C)GATACTTTGGTTTA(T/C)CACCCATCTGTT-3' and 5'-GTC-AAGTTC(T/C)TAGA(T/C)GGCTC(C/T)GC(C/T)GGCCGTGAGAAAGT-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 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 AgeI, 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 BglII-BamHI fragment containing PMP27 was isolated from N7 and subcloned into pUC19. The resulting plasmid was digested with XbaI and AgeI to liberate an internal region of the Pmp27 coding sequence. This region was replaced by the URA3 XbaI-XmaI fragment to generate the plasmid PMP27KO. This resulted in a PMP27 gene in which 44% of the open reading frame, from base 55 to 367 (see Fig. 1), was replaced by URA3. PMP27KO was digested with NgoMI, SacI, and AatII (digestion of the vector sequence with AatII was necessary since the vector and insert fragments were the same size), and the resultant 2.9-kbp fragment was used for transformation of MMYODIP1 by the lithium acetate method (Ito et al., 1983).

Uracil prototrophs were isolated, and Southern blots were performed on eight of the diploids to confirm that homologous integration had occurred. They were sporulated and haploid tetrads were analyzed again. As before, uracil prototrophy cosegregated with the PMP27 disruption. The phenotypes of strains 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 the BamHI/ClaI sites of pRS313 (CEN6, HIS3) (Sikorski and Hieter, 1989). This fragment was sufficient for oleate-inducible expression of Pmp27 (data not shown). pRS313 alone served as the negative control. For the overexpression plasmid YEplac112+PMP27, the PMP27 fragment was removed from pRS27 with BamHI and SalI and was ligated into the BamHI/SalI sites of YEplac112 (2 μm, TRP1) (Gietz and Sugino, 1988). YEplac112 served as the high-copy plasmid negative control. All four plasmids were singly transformed into strain 3B and expression of Pmp27 was checked by immunoblotting.

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,

(NH$_2$-CGDEHEDHKKVLGK-COOH), was synthesized (Clive Slaughter, UT Southwestern, Dallas, TX). This peptide was coupled to keyhole limpet hemocyanin using m-maleimidobenzoyl-N-hydroxysulfoxuccinimide 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 Towbin et al. (1979) using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) for detection. The thiolase antibody for the immunoblots 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; antithiolase 1:40,000; anti-porin (gift of G. Schatz, Biocenter, Basel) 1:1,000. Molecular weight standards were from Bio-Rad Labs (Hercules, CA). Quantitation of X-ray films was performed on a Zenith Soft Laser Scanning Densitometer.

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-309                                                              CCAAGACCA  -301

-300   GACCAGGAATATCTATATTGAAATTTACAAGGAAACTCTGTCCCATCTGCTAGGACATCA  -241

-240   TCAATGTCATAGTAACCCATCCTTTCTATGCTTGCCCTTCTATTGTAAAAATATAATACC  -181
                                          ------------->  <---
-180   AAGAACAAAGTTGGCATCAATTCCATTGCGATTTTCACTACGCGTCGGCATTTATCTCGG  -121

-120   AGAACCCGAAGCGATGGGTATATCATTTATAGATATAAAGGGAAGTGACTCCAACAACT   -60

-61    GAAAAACCGTTGTCTCCATCTACTACTTCAAAGACTTCATCAAGTAATAGTATAATCAAT    -1

  1    M  V  C  D  T  L  V  Y  H  P  S  V  T  R  F  V  K  F  L  D    20
  1    ATGGTCTGTGATACACTGGTATATCATCCCTCCGTGACGAGATTCGTCAAATTTCTAGAT   60

 21    G  S  A  G  R  E  K  V  L  R  L  L  Q  Y  L  A  R  F  L  A    40
 61    GGCTCAGCTGGCAGAGAAAAGGTTCTCAGATTACTGCAGTATTTAGCAAGATTTTTAGCA  120

 41    V  Q  N  S  S  L  L  A  R  Q  L  Q  A  Q  F  T  T  V  R  K    60
121    GTACAGAACTCATCTCTCTTGGCCAGGCAATTACAAGCTCAATTTACCACAGTCAGAAAA  180

 61    F  L  R  F  L  K  P  L  N  H  L  Q  A  A  A  K  F  Y  D  N    80
181    TTTCTGAGGTTTCTGAAGCCTTTAAACCACTTGCAGGCAGCTGCTAAATTCTACGATAAC  240

 81    K  L  A  S  D  N  V  V  R  V  C  N  V  L  K  N  I  F  F  A   100
241    AAGTTGGCCAGCGACAACGTCGTTAGAGTCTGCAATGTTTTGAAAAACATCTTCTTTGCT  300

101    A  Y  L  S  L  D  Q  V  N  L  L  R  I  L  K  V  I  P  V  T   120
301    GCATATTTGTCGTTAGATCAAGTCAATCTTTTGAGAATTTTGAAAGTGATTCCTGTAACC  360

121    V  L  T  G  K  K  I  P  R  W  S  N  W  C  W  L  F  G  L  L   140
361    GTTCTTACCGGTAAGAAAATACCTCGCTGGTCCAATTGGTGTTGGCTGTTCGGCCTCCTA  400

141    S  G  L  A  M  D  L  R  K  I  Q  T  S  H  A  Q  I  A  A  F   160
401    AGTGGTCTGGCTATGGATCTTCGTAAGATCCAAACATCTCATGCCCAGATTGCTGCGTTT  460

161    V  K  A  K  S  Q  S  Q  G  D  E  H  E  D  H  K  K  V  L  G   180
461    GTCAAGGCAAAATCACAAAGCCAAGGCGATGAGCATGAGGATCACAAGAAGGTACTAGGG  540

181    K  A  Y  Q  D  R  Y  T  A  L  R  R  L  F  W  D  A  A  D  S   200
541    AAGGCATACCAAGACAGGTACACCGCGTTAAGAAGACTATTCTGGGATGCTGCAGATTCG  600

201    P  I  V  L  N  N  L  G  Y  L  S  S  N  E  E  Y  V  A  L  S   220
601    TTCATCGTCCTCAACAACCTAGGGTACTTGTCTAGCAACGAAGAGTATGTTGCGCTATCC  660

221    G  V  V  T  S  I  L  G  M  Q  D  M  W  K  A  T  *            236
661    GGTGTTGTCACATCTATCCTTGGTATGCAAGACATGTGGAAAGCTACATAGCTTTCTTTT  720

721    CATCTTTTTTTTTTATTTGGCTATTCTCCGCTTATGTTTGATTCGACCCTTCTTTATAAT  780

780    TTATTTCTTATGTATATGACCCAATGTTCCATGTCCATACCTGTATAAGATTCTGAGTGA  840

841    CAATAGTTCTTGTTATCCGCACCATTAATAACTAACTAACTATCTTTAAAGTAAAACTGG  900
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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 oleate 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.
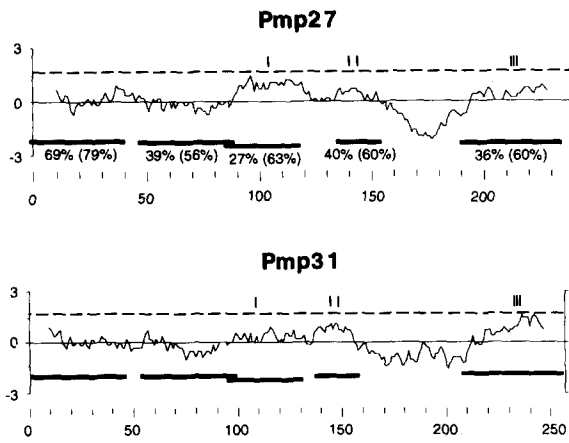
Figure 2. Pmp31 and Pmp27 are related sequences. Kyte-Doolittle hydropathy plots (Kyte and Doolittle, 1982) of Pmp31 from *Candida boidinii* and Pmp27 from *S. cerevisiae* are shown. The window of analysis is 19 amino acids. The dashed lines indicate the threshold for theoretical membrane-spanning domains. *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 μg 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 Gilbert, 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 0.1× 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 temperature. 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 immunochemistry, 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 performed on ultrathin sections with specific polyclonal antibodies against thiolase (gift of Wolf Kunau, University of Böchum, Germany) and gold-coupled goat-anti–rabbit antibodies by the method of Slot and Geuze (1984).
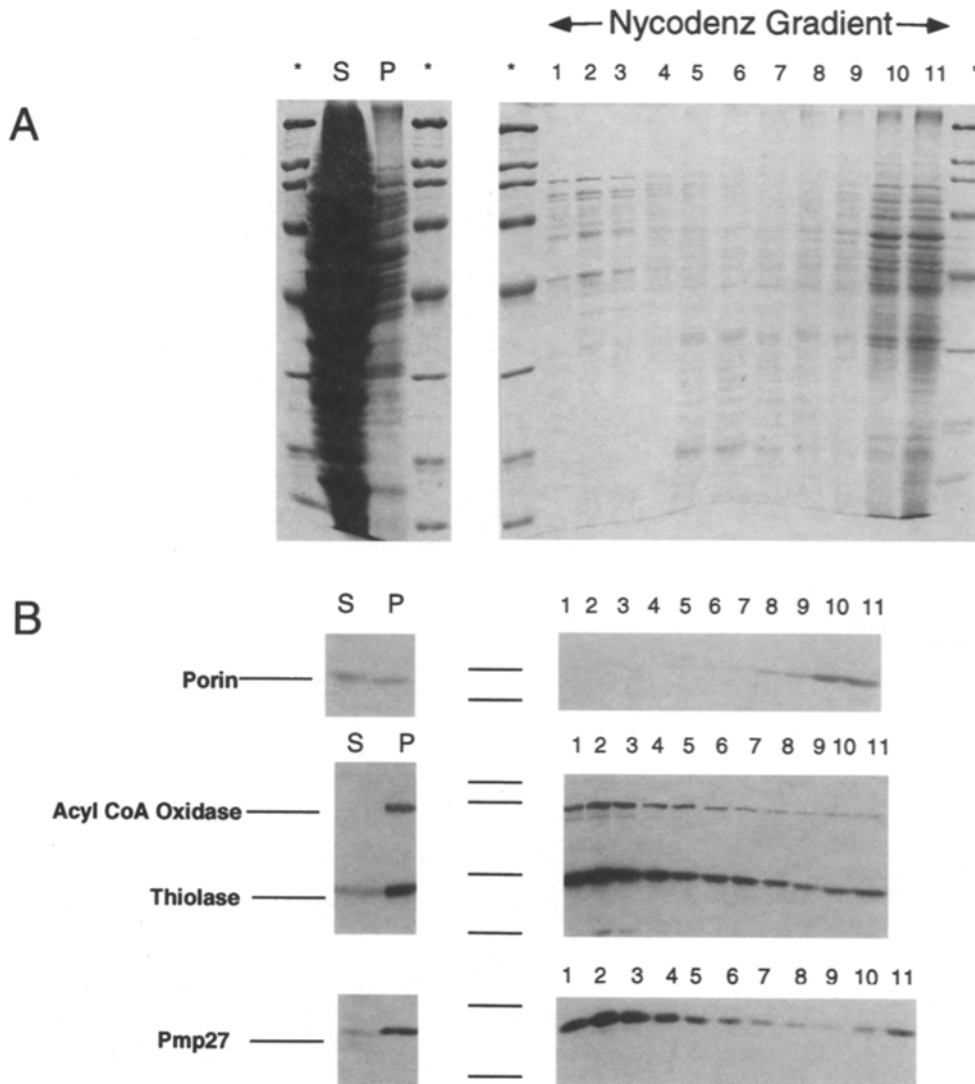

Figure 3. Pmp27 is a peroxisomal protein. (*S* and *P*) Supernatant and pellet resulting from a 25,000-*g* centrifugation; 0.5% of each was loaded on gels. Asterisks denote molecular mass standards. Numbers signify fractions from the Nycodenz gradients, from bottom to top; 20 μl of each fraction were loaded. (*A*) Coomassie-stained gel. Molecular masses of the standards are 200, 116, 97, 66, 45, 31, 21.5, and 14.4 kD. (*B*) Immunoblot from a gel identical to the one in *A*. Acyl CoA oxidase and thiolase were blotted together. Horizontal lines to the right of the blot denote standards as follows: the porin immunoblot: 35.1 and 29.7 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 boidinii

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 Pmp24 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 biodinii*. 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 AOU-1 (Sakai, Y., and J. M. Goodman, manuscript in preparation). These proteins are abundant in peroxisomal membranes of *C. boidinii*. 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 hydropathy 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-

nent of peroxisomal membranes (McCammon et al., 1990), its cellular distribution was not addressed. To perform localization experiments, we first prepared an antibody against a peptide derived from a sequence between patches II and III (amino acids 169-181). We then used this reagent to immunoblot organellar fractions from cells cultured in oleate (Fig. 3). A 25,000-g pellet, containing mainly mitochondria and peroxisomes (McCammon et al., 1990), was first prepared. The recovery of Pmp27 in this fraction, as determined by densitometry of immunoblots, was ~80% compared to the whole cell lysate. Organelles in this fraction were separated on a Nycodenz gradient. It can be seen that Pmp27 cofractionates with the peroxisomal enzymes acyl CoA oxidase and 3-ketoacyl thiolase, and is well separated from mitochondrial porin. These data suggest that Pmp27 is exclusively localized to peroxisomes.

We have recently shown that Pmp27 is not removed from peroxisomes by incubation at pH 8.5, a condition in which matrix proteins are released (McNew and Goodman, 1994). To further probe the relationship of Pmp27 to the peroxisomal membrane, we first determined its behavior in Triton X-114 (Fig. 4 *A*). A solution of this detergent partitions into two phases upon warming, traditionally separating hydrophobic membrane-spanning proteins from hydrophilic proteins. When organelles from a 25,000-g pellet were incubated with Triton X-114, virtually 100% of extracted Pmp27 partitioned into the detergent-rich phase after warming. However, some of Pmp27 was found to be insoluble, for unknown reasons. In contrast, thiolase partitioned into the detergent-poor (aqueous) phase, as expected. The behavior of Pmp27 in this detergent supports the hydrophobic nature of this protein and suggests an intimate association with the peroxisomal membrane.

Previous data had indicated that at least a fraction of Pmp27 was resistant to extraction by sodium carbonate, pH 11.5 (McCammon et al., 1990), a treatment that removes peripheral proteins from membranes (Fujiki et al., 1982). In this previous experiment, membranes were first purified from isolated peroxisomes. Without antibodies, however, it was difficult to determine the extent of extraction. We repeated the carbonate treatment using the Pmp27 antibodies and the crude 25,000-g organellar pellet. To our surprise, we found that almost all of the protein was extractable with car-
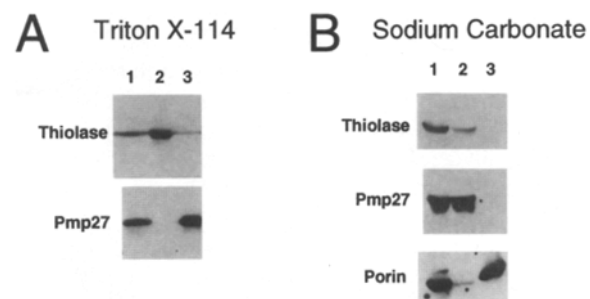

Figure 4. Pmp27 is hydrophobic but is probably not an integral membrane protein. (*A*) A 25,000-g organellar pellet was extracted with Triton X-114. The insoluble fraction (lane *1*), the aqueous phase (lane *2*), and the detergent phase (lane *3*) were subjected to SDS-PAGE and immunoblotting. (*B*) A similar 25,000-g pellet was subjected to extraction with 100 mM sodium carbonate. Starting material (lane *1*), extractable fraction (lane *2*), and inextractable fraction (lane *3*) were analyzed as in *A*.
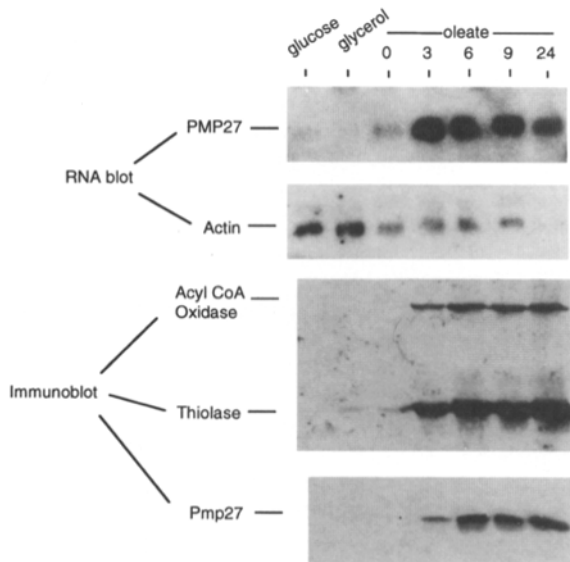
Figure 5. PMP27 is induced by oleate. Total RNA and protein were isolated from cells growing in the indicated carbon source. Cultures in glucose and glycerol were harvested in mid-logarithmic phase; cells in oleate medium were harvested after the indicated hour in the medium. Approximately 10 μg of RNA was electrophoresed on a glyoxyl agarose gel and probed with a fragment from *PMP27* or from the yeast actin gene as indicated. Protein, 30 μg per lane, was subjected to SDS-PAGE and immunoblotted with antibodies against the indicated proteins.

bonate (Fig. 4 *B*). In contrast, mitochondrial porin, a multiple membrane-spanning protein of the outer membrane, was resistant to extraction. The results of this experiment were reproducible. When we extracted highly purified peroxisomal membranes with carbonate, we found that 50% of Pmp27 was now inextractible, consistent with the previous finding (data not shown). We believe that the extensive manipulations to isolate peroxisomal membranes may have led to some artifactual inextractibility of Pmp27. We conclude that Pmp27 is probably not anchored in a transmembrane configuration, although it is certainly tightly associated with the peroxisomal membrane.

The Transcription of PMP27 Is Induced 100-fold by Oleate

The peroxisomal location of Pmp27 suggested that the protein may be inducible by oleate. We found this to be true (Fig. 5). *PMP27* mRNA could be detected in cells growing on glucose or glycerol as a 1,150-base band on Northern blots, but its concentration was very low. A comparison of the intensity of these two bands suggested that the gene was subject to only mild glucose repression. Within 3 h after incubation in oleate-containing medium, however, *PMP27* mRNA was already at 95% of maximal concentration as determined by radioanalytic imaging. This substrate was able to induce the mRNA approximately 100-fold, compared to levels in glycerol.

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, raffinose, 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 organellar pellets in all four strains. In contrast, Pmp27 is only detected in strains
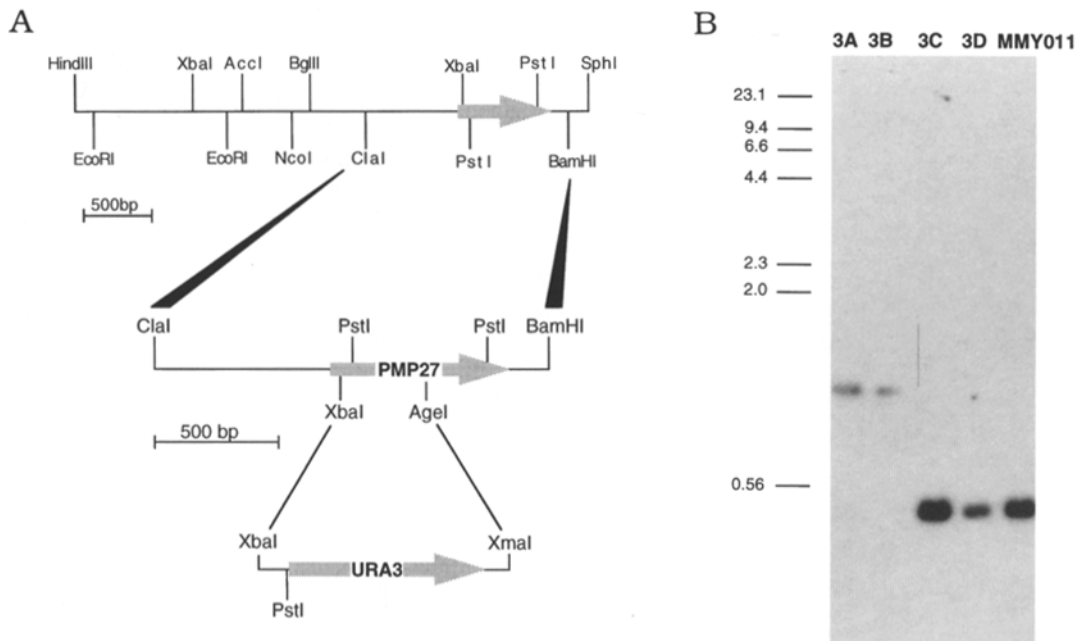
A

B

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. AgeI and XmaI 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.
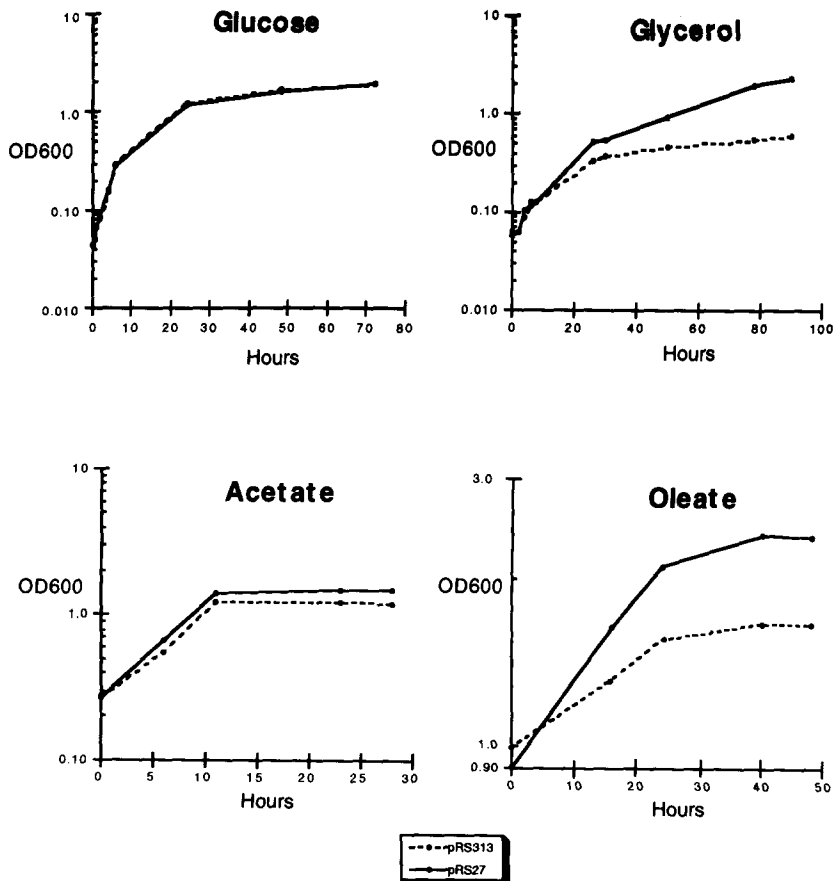

Figure 7. The *PMP27* disruption affects growth in glycerol, acetate, and oleate media. The disruption strain 3B was transformed with either pRS313 (*dashed lines*) or pRS27 (containing *PMP27, solid lines*) and cultured in the media as indicated. Growth was monitored by light scattering at 600 nm. For 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.
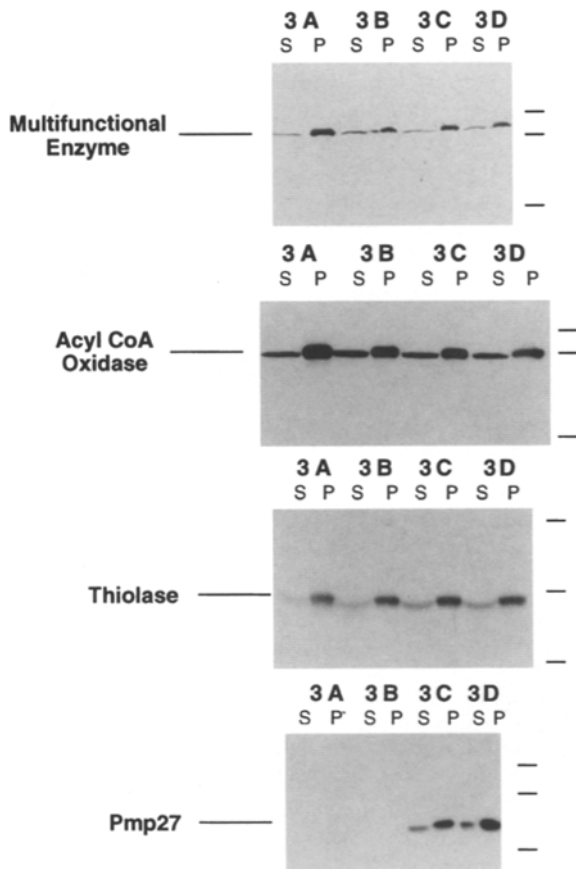
3A 3B 3C 3D
S P S P S P S P

Multifunctional
Enzyme

3A 3B 3C 3D
S P S P S P S P

Acyl CoA
Oxidase

3A 3B 3C 3D
S P S P S P S P

Thiolase

3A 3B 3C 3D
S P S P S P S P

Pmp27

Figure 8. PMP27 is not required for the import of peroxisomal matrix proteins. Strains from Tetrad 3 were grown in YPGOT, and 25,000 g organellar pellets and the corresponding supernatants were obtained. One percent of each sample was subjected to SDS-PAGE and immunoblotting with the indicated antibodies. Lines of standards are as indicated. *Multifunctional enzyme*, 142.9, 97.2, and 50 kD; *Acyl CoA oxidase*, 142.9, 97.2, and 50 kD; *Thiolase*, 97.2, 50, and 35.1 kD; *Pmp27*, 35.1, 29.7, and 21.9 kD.

3C and 3D, as expected. The minor fraction of all proteins, including Pmp27 in the supernatants may reflect breakage of some peroxisomes during fractionation. These data suggest that Pmp27 is not required for assembly of matrix proteins.

To confirm that the β-oxidation enzymes in the disruption were indeed in peroxisomes, the organelles in strains 3B-(pRS27) and 3B(pRS313) were fractionated on Nycodenz gradients. The gradient profiles were identical by protein 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-1 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, Pmps31-32 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 extractibility of Pmp27 from a crude organellar 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 inextractibility of Pmp27 with this reagent. We believe that the
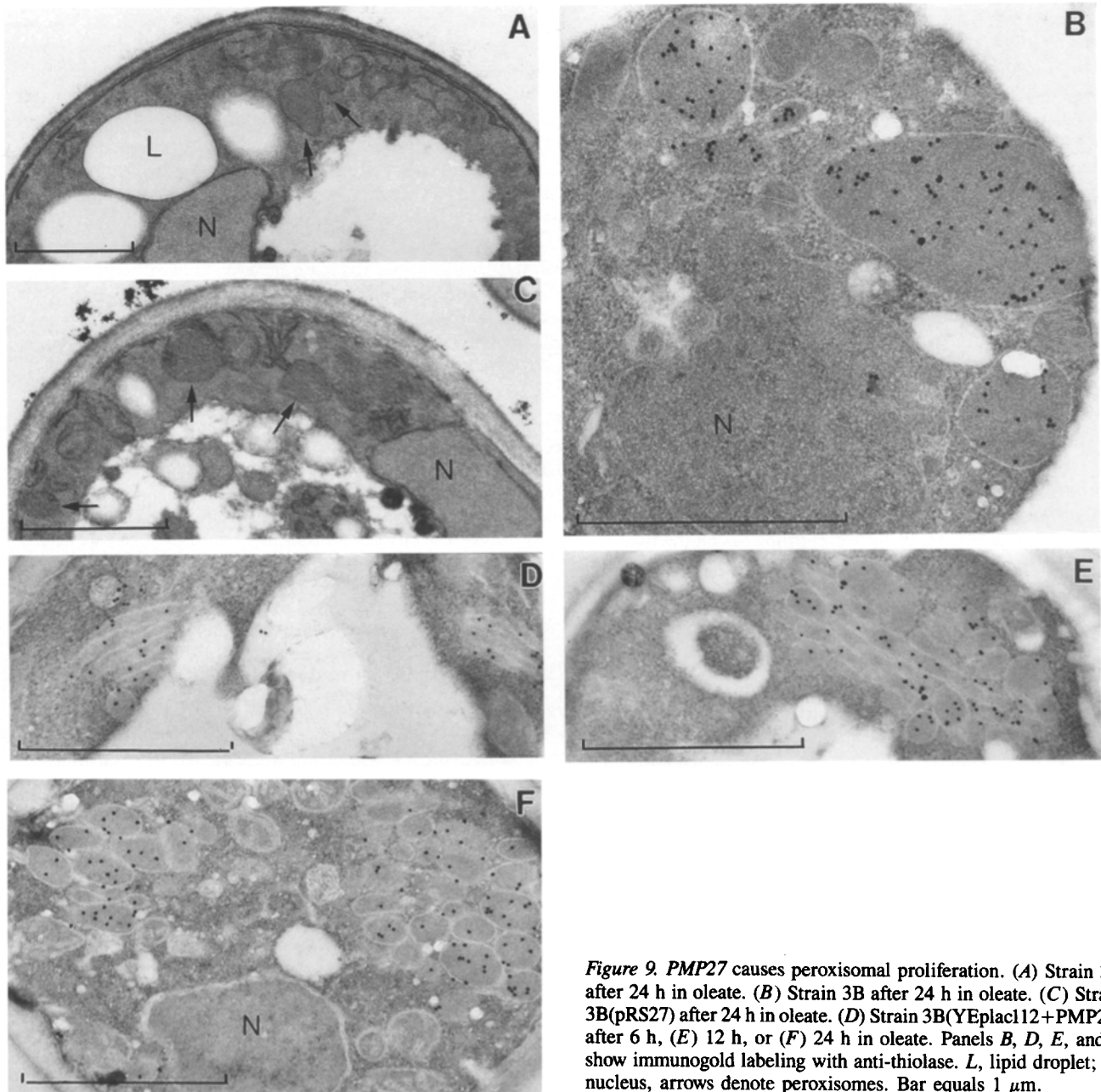
Figure 9. PMP27 causes peroxisomal proliferation. (*A*) Strain 3C after 24 h in oleate. (*B*) Strain 3B after 24 h in oleate. (*C*) Strain 3B(pRS27) after 24 h in oleate. (*D*) Strain 3B(YEplac112+PMP27) after 6 h, (*E*) 12 h, or (*F*) 24 h in oleate. Panels *B*, *D*, *E*, and *F* show immunogold labeling with anti-thiolase. *L*, lipid droplet; *N*, nucleus, arrows denote peroxisomes. Bar equals 1 μm.

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 con-

trol 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 organellar 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.

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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