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Redox-sensitive Homodimerization of Pex11p: A Proposed Mechanism to Regulate Peroxisomal Division

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Abstract. Pex11p (formerly Pmp27) has been implicated in peroxisomal proliferation (Erdmann, R., and G. Blobel. 1995. *J. Cell Biol.* 128: 509–523; Marshall, P.A., Y.I. Krimkevich, R.H. Lark, J.M. Dyer, M. Veenhuis, and J.M. Goodman. 1995. *J. Cell Biol.* 129: 345–355). In its absence, peroxisomes in *Saccharomyces cerevisiae* fail to proliferate in response to oleic acid; instead, one or two large peroxisomes are formed. Conversely, overproduction of Pex11p causes an increase in peroxisomal number. In this report, we confirm the function of Pex11p in organelle proliferation by demonstrating that this protein can cause fragmentation in vivo of large peroxisomes into smaller organelles. Pex11p is on the inner surface of the peroxisomal membrane. It can form homodimers, and this species is more abundant in mature peroxisomes than in proliferating organelles. Removing one of the three cysteines in the protein inhibits homodimerization. This cysteine 3 → alanine mutation leads to an increase in number and a decrease in peroxisomal density, compared with the wild-type protein, in response to oleic acid. We propose that the active species is the "monomeric" form, and that the increasing oxidative metabolism within maturing peroxisomes causes dimer formation and inhibition of further organelle division.

THE division of organelles is a basic cell function. It can be useful to think of organellar division as either constitutive or regulated, borrowing the nomenclature from the secretion field. Constitutive division is required to maintain organelle number and volume as cells divide and organelles age and undergo autophagy. In contrast, organelles can undergo regulated division in response to external signals or to internal programming. Thus, exposure to barbituates or overexpression of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase causes the proliferation of the endoplasmic reticulum (Jingami et al., 1987; Michalopoulos et al., 1976).

Peroxisomes are also exquisitely sensitive to external signals. In animal cells, various hypolipidemic drugs and plasticizers cause proliferation of this organelle (Lock et al., 1989), probably as downstream events upon activation of peroxisome proliferator-activated receptor-α (Lee et al., 1995). Although not all peroxisomal enzymes are induced by these agents, there is a severalfold increase in organelle number which accommodates an increased capacity for fatty acid β-oxidation (reviewed in Green, 1995).

Peroxisomal number and size are regulated to an even stronger extent in the methylotrophic yeasts which, for many years, have been model systems for peroxisomal assembly. For example, *Candida boidinii* grown on glucose medium contain only a few very small peroxisomes which undergo constitutive division as cells divide. When these cells are grown in the presence of either methanol, fatty acids, or *D*-alanine, however, there is massive peroxisomal proliferation, such that 30–50% of cell volume becomes comprised of peroxisomes, either clustered or separated (Goodman et al., 1990). The enzyme content of these organelles depends on growth substrate, because these compounds are directly utilized for peroxisomal metabolism, and the enzymes of different pathways are tightly regulated by the growth substrate (Veenhuis and Harder, 1987).

In contrast to matrix proteins, peroxisomes of *C. boidinii* contain membrane proteins that are induced by all proliferation substrates (Goodman et al., 1990). One such protein is Pex11p, also termed peroxin 11. (This protein was originally termed Pmp31 and Pmp32 to designate two nearly identical isoforms in *C. boidinii* [Goodman et al., 1986; Moreno et al., 1994], and later Pmp30A and B [Sakai et al., 1995]). The orthologue in *Saccharomyces cerevisiae* was termed Pmp24 (McCammon et al., 1990), later Pmp27 (Erdmann and Blobel, 1995; Marshall et al., 1995). The proteins in these two yeast species (CbPex11p and ScPex11p) can cross-complement (Sakai et al., 1995). Pex11p associates with the peroxisomal membrane but is releasable at high pH, indicating that it does not cross the bilayer (Goodman et al., 1986; Marshall et al., 1995).

We and others have shown that Pex11p functions in reg-

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ulated peroxisomal division (Erdmann and Blobel, 1995; Marshall et al., 1995; Sakai et al., 1995). Disruption of *PEX11* leads to one or two large organelles instead of several smaller ones in response to proliferators, suggesting that peroxisomal fission does not occur efficiently in the absence of this protein. The disruption strains grow more slowly in inducing substrates, possibly because they cannot segregate the large organelles. In contrast, there is no defect in peroxisomal protein import. Constitutive division of peroxisomes also seems unaffected; otherwise, a population of cells growing on glucose medium would soon be peroxisome free. In contrast to the deletion strains, overproduction of Pex11p leads to "hyperproliferation," in which the cell fills up with many more peroxisomes than normal (Marshall et al., 1995). The density of these organelles appears less than wild-type peroxisomes, consistent with our finding that there is no concomitant hyperinduction of matrix proteins (Marshall, P.A., unpublished data).

To understand how Pex11p regulates peroxisomal number and size, we have first determined the intraperoxisomal location of the protein. We also show that Pex11p can self-associate, and that this process correlates with functional inhibition. Conversely, a point mutation in Pex11p that disrupts self-association leads to hyperproliferation. We present a hypothesis to explain how peroxisomal metabolism may alter the oligomeric state of Pex11p, thereby regulating organellar division.

Materials and Methods

Strains and Culturing Conditions

TG1 cells (Sambrook et al., 1989) were regularly used for bacterial propagation of plasmids. All yeast expression experiments were performed using *S. cerevisiae* strain MMYO11α (McCammon et al., 1994), the derived strain 3B containing a disruption in *PEX11* (*PEX11::URA3*) (Marshall et al., 1995), or transformants of 3B described herein (Table I).

Yeast strains were cultured in oleic acid–containing medium as described in Dyer et al. (1996). Unless otherwise stated, cells were harvested after 16 h. For the experiments in which Pex11p expression was driven by the *GAL1-10* promoter, cells were precultured in SGd (0.67% yeast nitrogen base [Difco Laboratories, Inc., Detroit, MI], 3% glycerol, 0.1% dextrose) as usual and then incubated in Holland minimal medium with yeast

Table I. Strains Used in This Study

Strain	Genotype	Reference
MMYO11α	*MAT α ade 2-1 his3-11, 15 leu2-3, 112 trpl-1 ura 3-1 can1-100 Ole⁺*	McCammon et al., 1990
3B (="the Δ*pex11* strain")	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole⁺*	Marshall et al., 1995
3B (pMW46)	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole⁺* (*LEU2 CEN4*)	This study
3B (pMW46PMP27)	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole⁺* (*LEU2 CEN4 GAL1-10* promoter *PEX11 GAL7* terminator)	This study
3B (pMW46 & p27TGFPAKL)	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole⁺* (*LEU2 CEN4*) (2μ *TRP1 PEX11* promoter *GFPAKL PEX11* terminator)	This study
3B (pMW46PMP27 & p27TGFPAKL)	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole⁺* (*LEU2 CEN4 GAL1-10* promoter *PEX11 GAL 7* terminator) (2μ *TRP1 PEX11* promoter *GFPAKL PEX11* terminator)	This study
3B (pCEN-27-6HIS)	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole⁺* (*CEN6 TRP1 PEX11-6HIS*)	This study
3B (pRS27)	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole⁺* (*CEN6 HIS3 PEX11*)	Marshall et al., 1995
3B (pmp27C3A314)	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole+* (*CEN6 TRP1 pex11 C3A* mutant with native *PEX11* promoter & terminator)	This study
3B (pRS27 & p27T-GFP-AKL)	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole⁺* (*CEN6 HIS3 PEX11*) (2μ *TRP1 PEX11* promoter *GFPAKL PEX11* terminator)	This study
3B (pmp27C3A314 & pRS305GFPAKL)	*MAT α PEX11::URA3 ade 2-1 his3-11,15 leu2-3, 112 trp1-1 ura 3-1 can1-100 Ole⁺* (*CEN6 TRP1 pex11 C3A* mutant with native *PEX11* promoter & terminator) (2μ *LEU2 PEX11* promoter *GFPAKL PEX11* terminator)	This study

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extract and oleate (HMYO)[1] (semisynthetic medium with 0.1% oleic acid [van Dijken et al., 1976]) for 20 h to allow formation of large peroxisomes. Ultrapure galactose (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 0.1%, and the cells were incubated at 30°C for 6 h. The cells were then spun out of the galactose-containing medium and were placed into an equal volume of HMYO. Aliquots were taken at the indicated time points (see Figs. 1 and 2) after the addition of galactose and processed for electron and fluorescence microscopy as indicated below.

Organelle Fractionation and Preparation of Whole Cell Lysates

Preparation of crude organellar pellets and their fractionation on Nycodenz gradients were performed as described (Dyer et al., 1996). Whole cell lysates were prepared as before (Goodman et al., 1990). Protein concentrations of lysates were determined by the amido black procedure (Schaffner and Weissmann, 1973).

Protease Protection

Cells incubated in HMYO were converted to spheroplasts and osmotically lysed in 5 mM MES-NaOH pH 5.5 and sorbitol as described (McNew and Goodman, 1994) except that no protease inhibitors were added. Unlysed spheroplasts, nuclei, and other large particles were removed by centrifugation at 500 *g* to yield a lysate of ~2 mg/ml protein. For the experiment involving Triton X-100, proteolysis reactions consisted of 50 μl lysate, 5.5 μl 10% Triton X-100 or water, and 3 μl proteinase K to yield the mass of protease indicated (see Fig. 4). 1 ml of 20% TCA was added either immediately or after a 30-min incubation on ice to stop the reaction. For the freeze-thaw experiment, reactions consisted of 50 μl lysate and 3 μl proteinase K to yield the mass of protease indicated. The samples were then subjected to four cycles of freeze (using liquid nitrogen) and thaw. TCA was added immediately after freeze-thaw or after an additional 30-min incubation on ice.

All TCA-treated lysates were kept on ice at 4°C overnight, spun in a microfuge for 15 min at 4°C, washed twice in 1 ml acetone, and then resuspended in 50 μl 0.1 N NaOH containing 1% SDS. After the pellet was dissolved, 50 μl of Laemmli sample buffer was added, and the proteins were boiled and subjected to SDS-PAGE on 12% gels.

Chemical Cross-linking

Cross-linking was performed on 25,000 *g* organellar pellets or Nycodenz gradient fractions using bis [sulfosuccinimidyl] suberate (BS^3) (Pierce Chemical Co., Rockford, IL). The organellar pellet was resuspended in 50 mM Hepes, 5 mM EDTA, pH 8.0, and its protein concentration was determined by protein assay (Bio-Rad Laboratories, Hercules, CA). BSA was added to yield 1 mg/ml final protein concentration. Cross-linking was initiated by the addition of 10 μl cross-linker at the indicated concentrations to 40 μl of the resuspended pellet. The reaction was carried out for 1 h on ice and then terminated by the addition of 16 μl 4× SDS-PAGE sample buffer. The samples were boiled, resolved on a 9% SDS polyacrylamide gel, and subjected to immunoblotting with the Pex11p antibody.

For the cross-linking of the organelle fractions from Nycodenz gradients (see Fig. 8), gradient fractions were first assayed for total protein and Pex11p (by quantitative densitometry). They were then diluted to equalize the concentration of Pex11p and supplemented with BSA such that all samples had 40 μg total protein in 36 μl. To this volume, 4 μl of 500 mM Hepes pH 8.0 was added to raise the pH of each fraction to ~8.0. 10 μl of cross-linker (at different concentrations) was added. Samples were processed for immunoblotting as described above.

Two-dimensional Gel Electrophoresis

Fractions 1–4 from a Nycodenz gradient (the peroxisomal peak) were pooled and then spun at 57,000 *g* for 30 min in a centrifuge (TL 100; Beckman Instruments, Inc., Palo Alto, CA) to pellet the peroxisomes. Organelles were resuspended in 80 μl (final volume) 50 mM Hepes, 5 mM EDTA, pH 8, to yield a protein concentration of ~6 mg/ml. 10 μl of the reducible cross-linker 3,3′ dithiobis [sulfosuccinimidylpropionate] (DTSSP) (Pierce Chemical Co.) was added to each of two 40-μl aliquots of suspension to yield 2.5 nmol cross-linker/μg protein. Cross-linking proceeded for 1 h on ice, and was then terminated by the addition of SDS-PAGE sample buffer without reducing agent (the Tris base saturates the cross-linker). Solid DTT was added to the control aliquot to 100 mM final concentration and was incubated for 1 h at 37°C to reduce the crosslinker. The experimental aliquot was kept on ice during this time. Both samples were then boiled and subjected to SDS-PAGE on a 9% gel. Next, the lanes were removed and treated with 1× sample buffer containing 100 mM DTT for 1 h at 37°C. Each lane was then layered on top of a 1.5-mm 12% SDS gel and electrophoresed in the second dimension. Protein was detected by Coomassie blue staining. In a parallel and identical experiment, the 12% gel of each was transferred to nitrocellulose for immunoblot analysis.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed (Laemmli, 1970) with the stacking gel at pH 6.8, 4% acrylamide, and 9 or 12% separating gels at pH 9.2. Nonreducing SDS-PAGE was performed in the same way, but without β-mercaptoethanol in the sample buffer. Molecular weight standards were purchased from Bio-Rad Laboratories.

Proteins were transferred to pure nitrocellulose (Schleicher & Schuell, Keene, NH) (Towbin et al., 1979) and were blotted with the indicated antibody. Detection was carried out with ECL chemiluminescence (Amersham Corp., Arlington Heights, IL) according to the manufacturer's recommendations. The antithiolase antibody, a gift of Jon Rothblatt (Dartmouth Medical School, Hanover, NH) was used at 1:40,000, the affinity-purified peptide anti-Pex11p antibody (Marshall et al., 1995) was used at 1:1,000, and the anti-acyl coenzyme A oxidase antibody (McNew et al., 1993) at 1:500. Quantitation of immunoblots was performed with a computing densitometer, model 300A, and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) in the linear range of the film.

Plasmid Constructions

Construction of Pex11p Containing 6 Histidines at its Carboxy Terminus. Plasmid pKS27, which contains the ClaI-BamHI fragment of Pex11p in pBluescript KS- (Dyer et al., 1996), was digested with PstI to remove the majority of the Pex11p coding sequence. Religation of the vector piece produced clone pKS27ΔP, which contained a small region of the coding sequence (~250 bp) that was amenable to site-directed mutagenesis and sequencing. The 6-His tag sequence, a stop codon, and an EcoRI site (for diagnostic purposes) were introduced at the COOH terminus of Pex11p using site-directed mutagenesis (Kunkel et al., 1987) (oligo 27-6His: CAT GTG GAA AGC TAC ACA TCA CCA TCA TCA CCA TTA AGA ATT CCT TTC TTT TCA TCT T). The coding region and mutagenic additions were sequenced using primer 6H-seq (CCA ACA ACT GAA AAA CCG). This analysis revealed a short deletion at the NH_2 terminus of Pex11p. The correct sequence was obtained by subcloning a wild-type ClaI-PstI fragment into this clone. The rest of the Pex11p coding sequence was regenerated by subcloning the PstI fragment back into the middle of the Pex11p sequence. Orientation was verified by restriction mapping. The gene cassette was transferred to a yeast shuttle vector by subcloning a KpnI-SacI fragment into p27T-CAT-HA (Dyer et al., 1996). The final plasmid was called pCEN-27-6His (*CEN6, TRP1*).

Construction of GAL1-10 PEX11. Site-directed mutagenesis of the ATG of *PEX11* was performed with the oligonucleotide PMP24MUT-1 (GTA TCA CAG ACC ATG GTG ATT ATA CTA TTA C) to introduce a silent NcoI site. The mutagenesis was confirmed by sequencing. The gene was cut with NcoI, the end was blunted using Klenow, and the gene was cloned into the SmaI and BamHI sites of pUC18 (Sambrook et al., 1989). The *PEX11* gene was then removed with EcoRI and BamHI and placed into the vector pMW46 (*CEN4, LEU2*), a kind gift of Mark Walberg (UT Southwestern Medical Center, Dallas, TX). The *PEX11* sequence was placed behind the *GAL1-10* promoter and in front of the *GAL7* terminator. Expression of Pex11p was confirmed by growing the cells in galactose-containing media and immunoblotting for Pex11p. The empty pMW46 (*CEN4, LEU2*) vector was used as the negative control plasmid.

Construction of pex11p C3A. pKS27poly (Dyer et al., 1996) was cut with HindIII and BamHI and isolated to yield an empty vector which contained the *PEX11* promoter region. The *PEX11* coding sequence and terminator from the XbaI site to the BamHI site (missing codons 1 through 19) were removed from pKS27 (Dyer et al., 1996) and isolated. Two synthetic oligonucleotides, PMP27C3A (CTA GAA ATT TGA CGA ATC TCG TCA CGG AGG GAT GAT ATA CCA GTG TAT CTG CGA CCA TA) and PMP27C3A#2 (AGC TTA TGG TCG CAG ATA CAC TGG TAT ATC ATC CCT CCT TGA CGA GAT TCG TCA AAT TT), were annealed to generate a fragment that was compatible for three-frag-

1. *Abbreviations used in this paper*: BS^3, bis [sulfosuccinimidyl] suberate; HMYO, Holland minimal medium with yeast extract and oleate.

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ment ligation with the vector and Pex11p coding sequences. This ligation resulted in the generation of the intact Pex11p coding region except for a TGT to GCA mutation at codon 3, which changes cysteine to alanine. The mutation was confirmed by sequencing. The pex11p C3A gene cassette was transferred to a yeast shuttle vector by cloning a KpnI-SacI fragment into pRS314 (Sikorski and Hieter, 1989) (*CEN6, TRP1*). The final plasmid was called pmp27C3A314.

Fluorescence Microscopy

All strains expressed p27T-GFP-AKL (Dyer et al., 1996) to allow visualization of peroxisomes. For some experiments (see Figs. 1–3), cells also contained a *CEN* plasmid (pMW46) with *PEX11* under the control of the galactose *1-10* promoter (experimental) or the plasmid with no inducible gene (control). For the morphology and morphometry studies on the pex11p C3A mutant, cells contained plasmids pRS27 or pmp27C3A314. All cells were washed twice with water, and then fixed overnight in 0.5% glutaraldehyde and 4% paraformaldehyde, pH 7.6, at room temperature. Cells were spun out of the fixative solution, and were mounted in Fluoromount-G (Fisher Scientific, Plano, TX) before viewing on an Axioplan (Carl Zeiss, Oberkochen, Germany) with a fluorescein filter. Micrographs were taken on TMAX 400 film (Eastman Kodak Co., Rochester, NY).

Morphometry on galactose-induced Pex11p was done blindly on unmarked samples. Random fields of cells were scored for number of peroxisomes, 1–3, or 4 or more. At least 150 cells per sample were counted. Scalloping was defined as a wavy shape of the peroxisomal membrane. Peroxisomal fragmentation was defined as adhering clusters of small fluorescent organelles. Significance was determined using the *t* test.

Quantitation of peroxisomes in cells expressing wild-type Pex11p or the C3A mutant was determined blindly on unmarked samples. The number of peroxisomes per cell was counted in random fields. At least 500 cells per sample were examined. Significance was determined using *t* test.

Electron Microscopy

For the studies on galactose-induced Pex11p, cells were cultured as described for fluorescence microscopy. Aliquots were taken at the indicated time points (Fig. 2), the cells were washed twice with water and then fixed in 1.5% $KMnO_4$ for 10 min at room temperature. The fixed cells were washed twice with water, and were stained overnight in 1% uranyl acetate. The pellets were subjected to a graded ethanol series, and were finally embedded in pure epon. The epon was heated to 75°C for over 12 h. Ultrathin sections were cut using a diamond knife, placed on 100-Hex copper grids (Ted Pella, Redding, CA), and were poststained for 15 min in 3% uranyl acetate and then Renoyld's lead citrate for 3 min. Grids were viewed on a JEOL 1200 (JEOL USA, Inc., Peabody, MA) at 60 kV. For the studies on the C3A and wild-type control, the cells were harvested after 16 h in oleic acid and were treated as described above.

Results

Pex11p Causes Fragmentation of the Large Peroxisomes in the Strain Harboring the PEX11 Disruption

While earlier studies indicated that Pex11p was involved with peroxisomal proliferation, it was not clear if its effect was direct or indirect. To test whether Pex11p is sufficient for this process, we asked whether it can cause fission of the large peroxisomes in the strain containing the disruption of the *PEX11* gene (strain 3B, hereafter called the Δ*pex11* strain). For this purpose *PEX11* was placed under the control of the galactose-inducible promoter *GAL1-10* and was introduced into the Δ*pex11* strain on a low-copy plasmid. A parallel control strain that lacked the *PEX11* coding region on the plasmid was also constructed. Both strains contained green fluorescent protein fused to a peroxisomal targeting signal (AKL), which allowed visualization of peroxisomes. The morphology of peroxisomes seen by fluorography was confirmed by electron microscopy.

Cells were first cultured in oleic acid–containing medium to induce formation of the large peroxisomes previously reported for the Δ*pex11* strain (Fig. 1, *0 h*, and Fig. 2 *A*). Over 80% of cells contained 1–3 peroxisomes (Fig. 3 *A*), compared to 20 or more peroxisomes typically found in the wild-type strain (see Fig. 11 *C*). Galactose was then added at a low concentration to minimize artifacts caused by overexpression, such as possible toxicity of mistargeted proteins. Immunoblots indicated that the maximal level of Pex11p obtained under these conditions was ~75% of wild-type cells grown in oleic acid–containing medium (data not shown). After 6 h of exposure to galactose, many of the peroxisomes from cells synthesizing Pex11p had a "scalloped" appearance, as if blebbing of the peroxisomal membrane was occurring (Fig. 1). This was confirmed by electron microscopy (Fig. 2 *B*). Scalloping could also be observed in the control strain, but much less frequently.

By 14–16 h fragmentation of the large peroxisomes could easily be seen by fluorescence microscopy (Fig. 1). Electron micrographs showed peroxisomes of aberrant shapes that appeared to be fragmenting (Fig. 2 *C*). Morphometric analysis revealed that >40% of cells displayed these patterns at 16 h (Fig. 3 *B*), while no peroxisomes from the control strain appeared this way. This was accompanied by a dramatic increase in the number of separate peroxisomes per cell (Fig. 3 *A*).

Finally, by 24–27 h the large peroxisomes had mostly disappeared from the Pex11p-expressing cells and were replaced by smaller organelles (Fig. 1, and Fig. 2 *D*). By this time, 78% of the cells had four peroxisomes or more (Fig. 3 *A*). In contrast, the control strain still had large and few peroxisomes.

Since the presence of galactose is unlikely to induce other peroxisomal proteins, we conclude that the synthesis of Pex11p at physiological levels is sufficient to cause fragmentation of peroxisomes, and that peroxisomal fission is inhibited in its absence.

Pex11p Resides on the Inner Surface of the Peroxisomal Membrane

As a first step toward understanding the function of Pex11p in peroxisomal fission, we determined the orientation of the protein with respect to the peroxisomal membrane.

When purified peroxisomes are subjected to a high pH wash, at least half of the total Pex11p content remains in the membrane pellet, consistent with the hydrophobic nature of the protein (Erdmann and Blobel, 1995; Marshall, P.A., unpublished results). However, if a less purified membrane pellet is similarly extracted, 95% of the Pex11p is removed (Marshall et al., 1995). We interpret these data to indicate that Pex11p is not a transmembrane protein, and that some aggregation of the protein on the membrane may be occurring artifactually during organelle isolation on Nycodenz or sucrose gradients.

To determine whether Pex11p was facing the cytoplasm or the matrix, a yeast lysate (prespun at 500 *g* to remove unbroken cells and other large particles) was subjected to treatment with proteinase K (Fig. 4). Pex11p was completely resistant to protease concentrations at or below 3 μg/reaction, although we often saw degradation at higher

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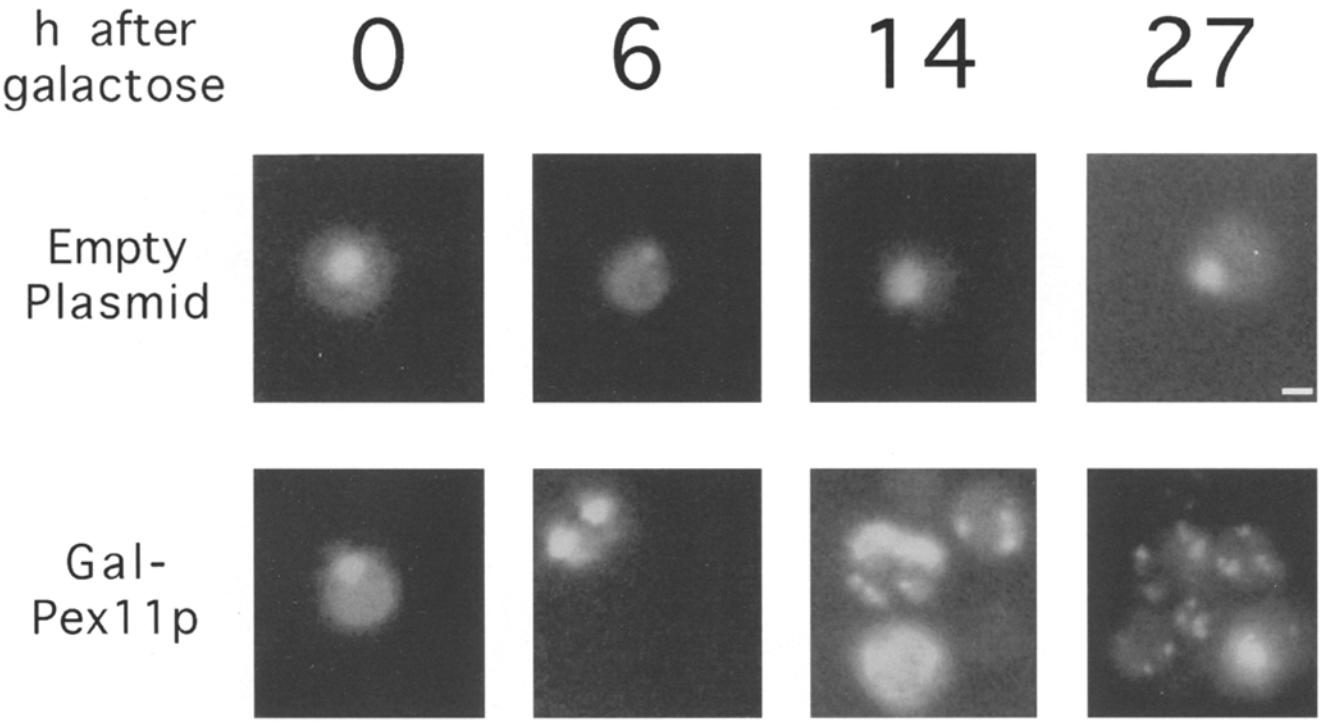

Figure 1. Pex11p can cause large peroxisomes to fragment. GFP-AKL was expressed in the Δ*pex11* strain carrying a plasmid containing *PEX11* driven by the *GAL1-10* promoter. The control lacked the *PEX11* coding region on the plasmid. Cells were incubated in oleic acid–containing media for 20 h and then galactose was added to a final concentration of 0.1% at $t = 0$. Cells were spun out of galactose and reinnoculated into HMYO at $t = 6$. Time points after galactose addition are as indicated. Bar, 1 μm.

concentrations. Since this occurrence was accompanied by noticeable clipping of the matrix protein thiolase, it probably represents partial access of protease to the matrix. In the presence of nonionic detergent, however, 3 μg protease was sufficient to degrade all of Pex11p. Thiolase was also clipped to a stable lower molecular weight fragment by this treatment, consistent with release of the matrix by detergent. We found that TCA precipitation of protein after protease treatment did not allow full recovery of Pex11p in the presence of detergent (Fig. 4, lanes containing Triton without incubation). The same recovery was observed, however, at all protease concentrations.

The higher sensitivity of Pex11p to proteolysis in the presence of detergent as compared with in its absence suggests that Pex11p resides on the inner surface of the peroxisomal membrane. To show that the increased sensitivity is not caused simply by the ability of detergent to directly interact with Pex11p, making it more accessible to protease, samples were subjected to freeze-thaw as an alternative to detergent to allow protease to enter the matrix. Even without the subsequent incubation, this treatment led to some degradation of protein (Fig. 4, *bottom right*). With incubation, there was total degradation of protein above 3 μg protease, while the matched control showed no effect at any concentration tested. These data indicate that Pex11p lies on the inner surface of the peroxisomal membrane.

Pex11p Forms Homodimers

The identification of protein–protein interactions can often provide information about function. For this reason we searched for partners of Pex11p, using chemical cross-linking.

An organellar fraction from cells induced with oleic acid was subjected to increasing concentrations of the amine-directed homobifunctional cross-linker BS^3. With increasing concentrations of cross-linker, a prominent 48-kD protein appeared, indicating that Pex11p was cross-linked to another protein of similar size (Fig. 5 *A*). The additional 55-kD band is a cross-reacting species that is often detected by the Pex11p antibody even in the absence of cross-linker.

The 48-kD complex may be a Pex11p homodimer or a heterodimer. To discriminate between these two possibilities, an organellar pellet that had been treated with a reducible amine cross-linker, DTSSP, was subjected to two-dimensional electrophoresis. The sample was first electrophoresed through a nonreducing gel, such that disulfide bonds, both in the middle of the cross-linker and between proteins, were kept intact. The lane was then removed and incubated with DTT. It was then applied sideways to a reducing SDS gel, run out, and stained with Coomassie blue. Most proteins fell along the diagonal, as expected, indicating that they were not disulfide bonded with another protein (Fig. 6 *A*). Pex11p, however, was resolved into two bands, one of which migrated as monomer (along the diagonal), and the other which migrated to the left, a result of the 48-kD complex (see arrow). Significantly, there were no other visible bands directly above or beneath the 48-kD–derived species. A control in which the reducing gels were used in both directions showed no 48-kD species, as expected (Fig. 6 *B*).

An immunoblot of a similar pair of gels confirmed the identity of the Pex11p-related bands (Fig. 6, *C* and *D*). It

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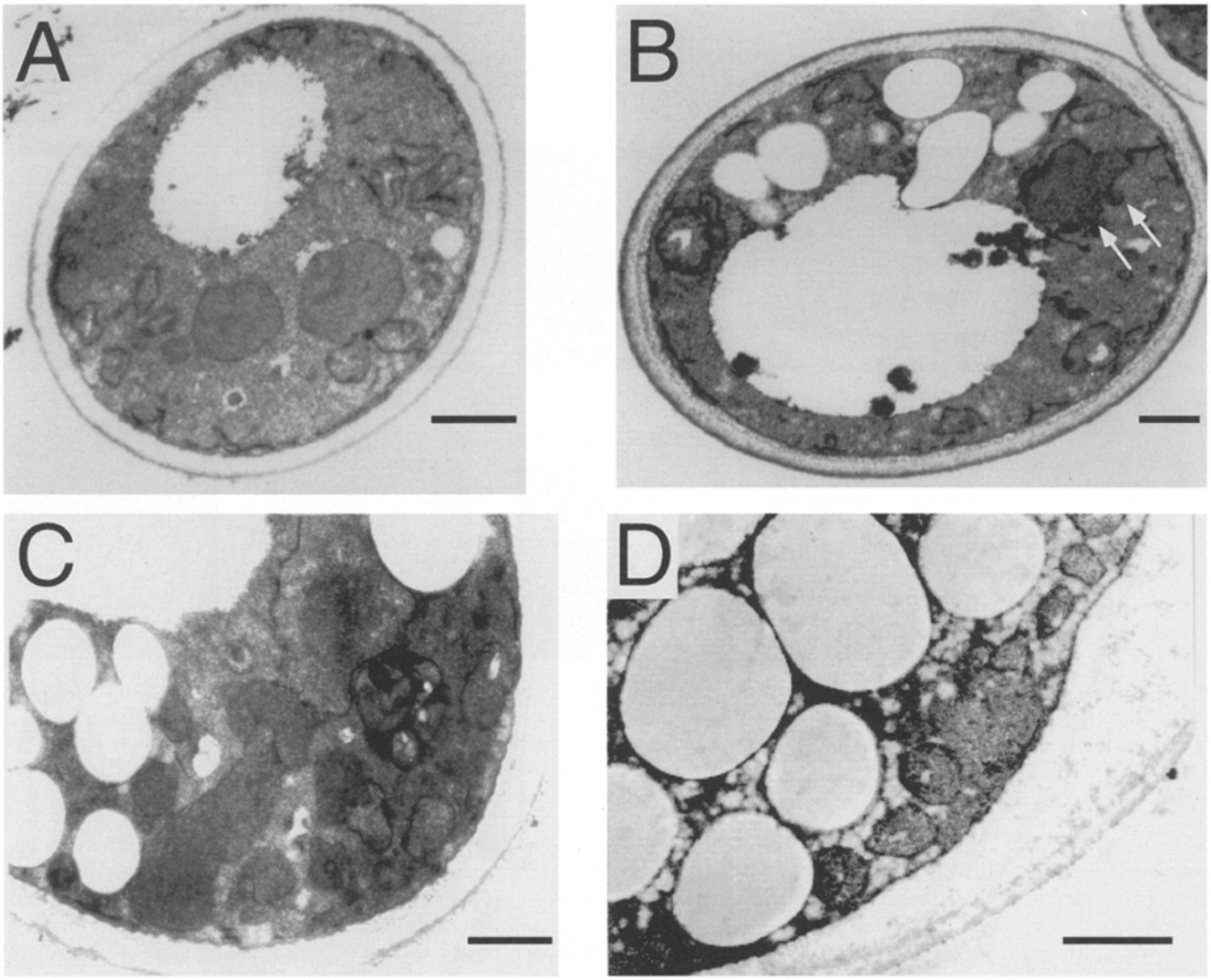

Figure 2. Electron microscopy confirms the Pex11p-induced budding of peroxisomes. (*A*) Cells of the Δ*pex11* strain carrying the inducible *PEX11* plasmid before the addition of galactose. (*B*) 6 h after the addition of galactose. Arrows point to peroxisomal buds. (*C*) 14 h after the addition of galactose. (*D*) 24 h after the addition of galactose. Bar, 500 nm.

also revealed minor species: a higher band corresponding to the fraction of Pex11p dimers that was not separated into monomers by the reducing treatment (*arrow*), and higher order Pex11p complexes (*arrowheads*).

The presence of a single stained protein to the left of the diagonal from Pex11p monomer (i.e., no other visible bands directly above or beneath; see arrow, Fig. 6 *A*) indicates either that Pex11p forms homodimers, or it forms heterodimers with a protein of virtually identical size. To rule out heterodimers, a cell strain was used in which a larger form of Pex11p (i.e., with six histidines attached to the carboxy terminus) was substituted for the wild-type protein in the Δ*pex11* strain. This Pex11p-6His protein can fully complement the growth phenotype of the disruption (Dyer, J.M., unpublished data). The two forms of Pex11p can be resolved on a gel (Fig. 7, *A* and *B*).

Two-dimensional gel analysis was applied as before to organelles from this strain. There was still only one Coomassie blue–stained band in the 48-kD region, corresponding to the Pex11p-6His protein (Fig. 7 *C*, *arrow*). These experiments show that Pex11p associates with itself rather than with another protein.

To simplify further discussion, we refer to the Pex11p species that is not cross-linked with BS^3, as "monomer," and the 48-kD species as "dimer." It should be noted, however, that "monomeric" Pex11p still may be associated with proteins not coupled by cross-linker, and the "dimeric" Pex11p may also exist as higher order structures. These issues are not easily resolved by size fractionation on columns or density gradients due to the hydrophobicity of the protein. We simply use these terms to indicate two distinct forms of the protein.

Mature Peroxisomes Have a Higher Dimer/Monomer Ratio than Immature Peroxisomes

Since protein–protein interactions often influence function, we asked whether peroxisomal proliferation was associated preferentially with either Pex11p monomers or dimers. First, we compared the degree of Pex11p cross-

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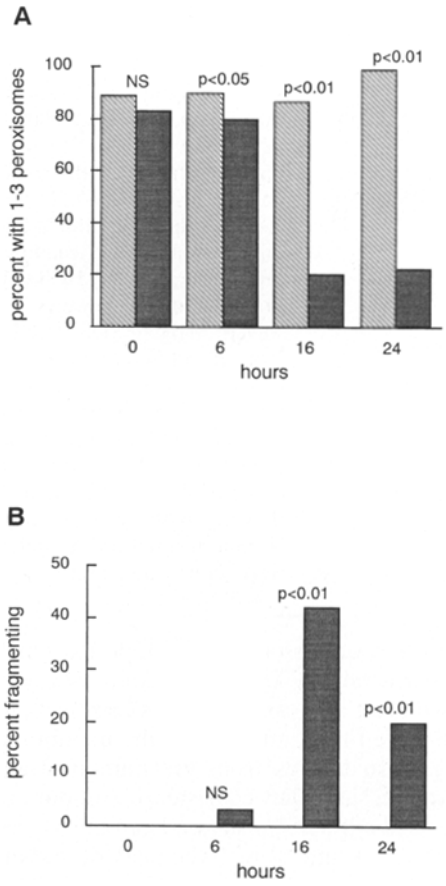

Figure 3. Morphometry on GFP-AKL expressing cells. Culture conditions were as described in legend to Fig. 1. Time points after galactose addition are as indicated. (*A*) Numbers of peroxisomes per cell. NS, not a statistically significant difference between the two numbers. p is the probability that the two numbers are not significantly different. (*B*) Percentage of cells that contained fragmenting peroxisomes. ▨, without Pex11p; ■, with Pex11p.

linking in peroxisomes at a stage of vigorous proliferation (8 h in oleic acid) compared with a later terminal stage (16 h). It can be seen that there is much more dimer formation at the later stage than at the early one (Fig. 5, *A* and *B*), suggesting that there are more dimers than monomers in mature peroxisomes. Pex11p at both time points could be extracted with carbonate but not salt (Marshall, P.A., unpublished observation), suggesting that both monomeric and dimeric Pex11p are tightly bound to the membrane.

The relationship of Pex11p monomers and dimers was further explored by determining the extent of dimer formation in peroxisomal fractions along a Nycodenz gradient. While mature peroxisomes (i.e., those predominating at steady state after complete oleic acid induction) sediment to the bottom of a Nycodenz gradient under standard conditions, immature peroxisomes (those first appearing shortly after oleic acid shift) are of lower density (Erdmann and Blobel, 1995 and Marshall, P.A., unpublished results). Thus a "smear" of peroxisomes is obtained from cells undergoing proliferation (Dyer et al., 1996). When we subjected various gradient fractions to cross-linking, under conditions where the concentration of Pex11p and total protein were kept constant, we observed the greatest extent of cross-linking in the dense organelles (Fig. 8, fraction *2*). Cross-linking was much lower in the lighter organelles (fractions *8* and *10*). It is noteworthy that Nycodenz fractions 6 and 10 (before any manipulation) contained an identical concentration of Pex11p, while cross-linking was vastly different (Fig. 8 *B*) The top fraction of the gradient may contain unbroken spheroplasts (McCammon et al., 1990), probably accounting for the increased signal in that fraction.

These data suggest that Pex11p monomers are preferentially associated with proliferating peroxisomes, whereas dimers are found in mature, fully functional organelles.

Inhibition of Dimer Formation Increases Peroxisomal Proliferation

Inspection of the sequences of Pex11p of *S. cerevisiae* and *C. boidinii* (Marshall et al., 1995; Moreno et al., 1994; Sakai et al., 1995) revealed an odd number of cysteine residues in each protein. Perhaps the formation of an intermolecular disulfide bond is involved with generation of Pex11p dimers. To test this possibility, a Nycodenz gradient obtained from cells undergoing active peroxisomal proliferation was electrophoresed on a nonreducing SDS gel. As shown in Fig. 9 *B*, the dimeric form of Pex11p can be easily seen, and it is enriched in fractions containing mature peroxisomes. This result parallels the cross-linking data presented above. Taken together, they support the idea that the monomeric form of Pex11p is involved with peroxisomal proliferation while the dimeric form, which is stabilized by disulfide bonds, is found in the mature organelle after termination of proliferation.

To determine if the putative redox-sensitive dimerization is simply a byproduct of peroxisomal metabolism or may be important for regulating the proliferation of organelles, we wanted to alter the cysteine residue participating in the crossbridge, thereby inhibiting dimer formation. The CbPex11p members have one cysteine at position 36, while ScPex11p has three cysteines at positions 3, 90, and 134. Although the exact position of the CbPex11p cysteine is not conserved in ScPex11p, it is located in an amphipathic region of the protein similar to the first cysteine in ScPex11p (Marshall et al., 1995) and is surrounded by a similar profile of amino acids (CbPex11p is LCRFLT(F/Y) while ScPex11p is VCDTLVY). Since cysteine has a very reactive side chain and the environments between the two proteins seemed to be conserved, we suspected that the single cysteine of CbPex11p and the first cysteine of ScPex11p might be available for intermolecular disulfide bond formation.

Therefore, the first cysteine residue of ScPex11p was mutated to alanine. If our hypothesis of active monomer is correct, then inhibiting dimer formation should enhance peroxisomal proliferation.

We expressed either the wild-type or the C3A mutant protein in the Δ*pex11* strain. Immunoblotting revealed that the C3A mutant was expressed to about half the level of the wild-type protein (Fig. 9 *A*). A cross-linking experiment revealed that the pex11p mutant could still form dimers, but levels were reduced to 30–40% of control levels as determined by scanning densitometry (Fig. 10). We saw no dimers, however, when samples were analyzed by nonreducing gels (Fig. 9 *C*).

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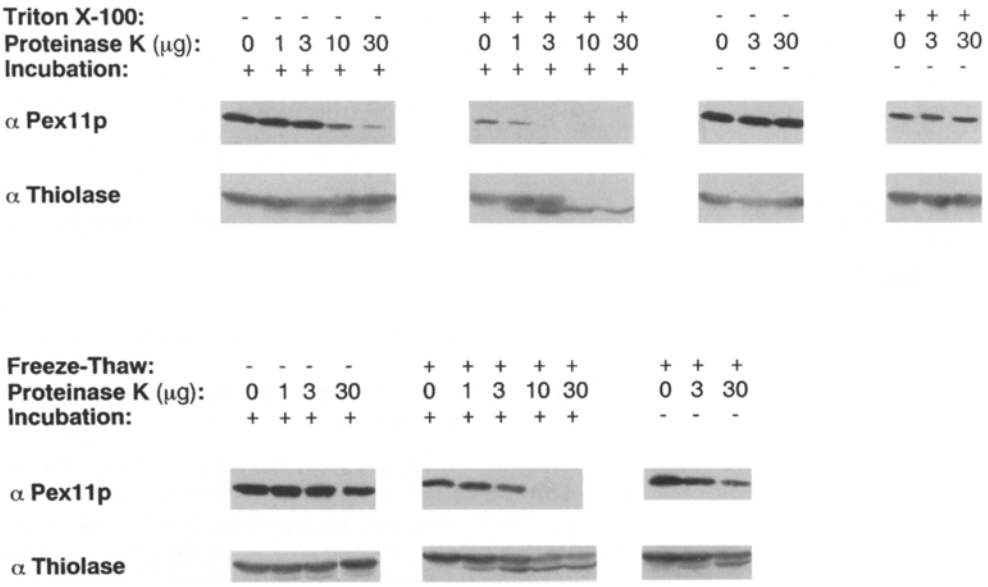

Figure 4. Pex11p is on the inside of the peroxisomal membrane. (*Top*) A 25,000-*g* pellet was subjected to protease treatment in the presence or absence of 1% Triton X-100 with or without a 30-min incubation on ice. Proteins were isolated by TCA precipitation and were resolved by SDS-PAGE. Pex11p and thiolase were detected by immunoblotting. (*Bottom*) An organellar pellet was subjected to protease treatment in the presence or absence of snap freezing and thawing with or without a 30-min incubation. Samples were then treated as above.

It is possible that some chemical cross-linking of monomers occurs, since Pex11p is the most abundant protein in the membrane and its concentration on the membrane surface may be very high in places. Our data show that C3 is indeed required for disulfide bonds between monomers, but we cannot conclude whether dimers can form even in the absence of C3. Regardless, this mutation caused an increase in the monomer/dimer ratio of Pex11p.

Consistent with our hypothesis that Pex11p dimerization inhibits proliferation, fluorescence microscopy of the C3A mutant revealed there were many more and smaller peroxisomes than in the control (Fig. 11, *A* and *B*). While most cells had between 21 and 40 peroxisomes in the control, there were well over 40 peroxisomes in most cells of the mutant strain (Fig. 11 *C*). Many peroxisomal tubules were also observed in the mutant (Fig. 11, *A* and *B*).

Blotting of fractions from Nycodenz gradients verified that the mutant protein was associating with peroxisomes (Fig. 9 *C*). The extractability of mutant and wild-type Pex11p proteins with salt and carbonate was identical, indicating that both were firmly attached to the membrane (data not shown). Peroxisomes from the mutant strain were slightly less dense, since they consistently (in four experiments) migrated to a lower density on isopycnic Nycodenz gradients (Fig. 12, *A* and *B*) with the peak of mutant peroxisomes in fractions 3 and 4 instead of 2 and 3. This behavior suggested that the peroxisomes harboring the mutant protein were less mature (Erdmann and Blobel, 1995). Additionally, the mitochondria of the pex11p C3A mutant migrated further in the gradient, a result which was reproducible. The mitochondria might be responding to a retrograde regulation signal (Liao and Butow, 1994) from the peroxisomes of the mutant.

We conclude that Pex11p monomers are the active species in promoting peroxisomal proliferation and that disulfide formation between monomers may terminate the proliferative process. We cannot yet conclude whether Pex11p dimers are weakly active, or completely inactive.

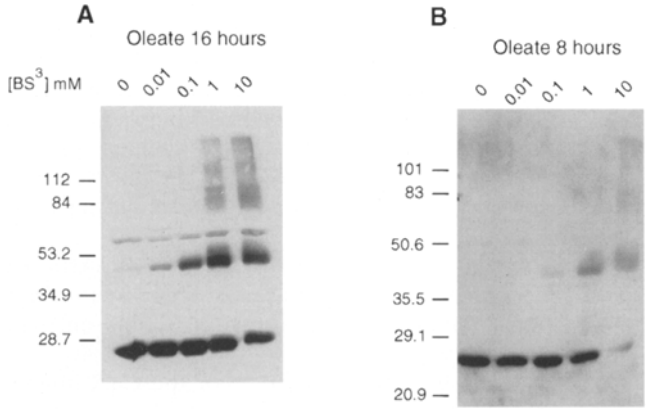

Figure 5. Pex11p is cross-linked to a protein of similar size. (*A*) An organellar pellet from cells grown in oleic acid for 16 h was subjected to increasing concentrations of the cross-linker BS^3. Proteins were resolved by SDS-PAGE and Pex11p was detected by immunoblotting. (*B*) An organellar pellet from cells grown in oleic acid for 8 h was subjected to increasing concentrations of the cross-linker BS^3. Proteins were treated as in (*A*).

Discussion

In this work we have provided further evidence that Pex11p promotes peroxisomal proliferation, that it performs its function from within the organelle, and that it undergoes homodimerization that correlates with the maturity of the organelle. We suggest that dimerization is an "off switch" for the protein. Consistent with this idea, inhibiting dimerization by mutating Cys 3 to Ala caused hyperproliferation of peroxisomes.

The data implicating a direct role of Pex11p in peroxisomal proliferation are now persuasive. The first indication of this effect was the morphological phenotype of the Δ*Scpex11* strain, namely large and often ill-shaped peroxisomes, far fewer in number compared with wild type, in oleate-grown cultures (Erdmann and Blobel, 1995; Marshall et al., 1995). A similar phenotype was seen in the Δ*Cbpex11* strain (Sakai et al., 1995). Although this pheno-

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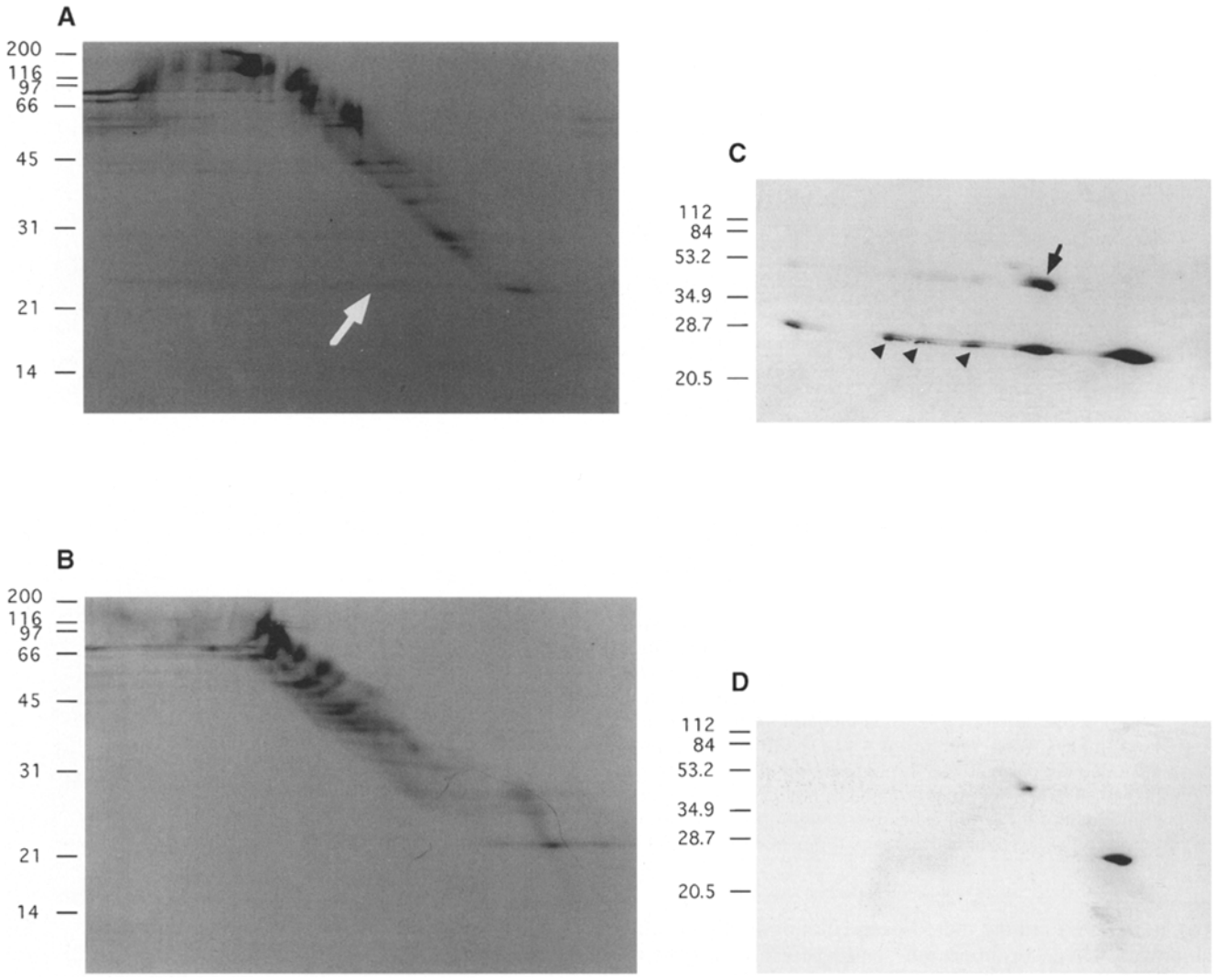

Figure 6. Pex11p can interact with a protein of virtually the same size. (*A*) Nycodenz-purified peroxisomes were cross-linked with the reducible cross-linker, DTSSP, and were resolved by nonreducing SDS-PAGE. The lane was removed, reduced, applied to a reducing gel, and electrophoresed in the second dimension. Proteins were stained with Coomassie blue. The arrow indicates Pex11p migrating off of the diagonal. (*B*) As in *A*, but the sample was reduced before electrophoresing in the first dimension. (*C* and *D*) Immunoblots with anti-Pex11p of gels similar to *A* and *B*, respectively. Arrow indicates nonreduced dimer, arrowheads indicate tetramer, hexamer, and octamer forms of Pex11p.

type is somewhat reminiscent of the slightly larger peroxisomes in mammalian cells lacking acyl-CoA oxidase (Poll-The et al., 1988), suggesting that metabolic defects can indirectly enlarge peroxisomes, further evidence indicates that the role of Pex11p is a direct one. First, there are many more peroxisomes in a Pex11p overproducer (Marshall et al., 1995). Also, CbPex11p is induced by three diverse carbon and nitrogen sources (Goodman et al., 1990), making it unlikely that this protein performs a distinct metabolic step. In addition, Pex11p is the most abundant peroxisomal membrane protein in *S. cerevisiae*, further suggesting a nonmetabolic role (McCammon et al., 1990). Finally, the experiment shown here, that Pex11p causes large peroxisomes to fragment into smaller organelles, argues most convincingly that this protein is acting directly in regulated organellar division.

We report two forms of Pex11p, monomer and dimer, and show that there is a correlation between peroxisomal density (probably indicating maturity) and monomer/dimer ratio. If we simply alter this ratio, which is accomplished in the C3A mutant, peroxisomal number dramatically increases. How might the organelle switch off further proliferation during its development? We hypothesize that the environment inside the peroxisome dictates the state of Pex11p. We have shown in *C. boidinii* that peroxisomal fission and a rapid increase in surface area of the peroxisomal membrane precedes synthesis and import of matrix enzymes (Veenhuis and Goodman, 1990). These early events may be driven by Pex11p acting as the monomer. Once import of matrix proteins begins, there is no significant increase in peroxisomal number, only in size of individual organelles (Veenhuis and Goodman, 1990). The oxidative metabolism that occurs once matrix proteins are imported and become functional may lock Pex11p into the dimer state by generating a cystine crossbridge, preventing further peroxisomal fission. Preventing this covalent link-

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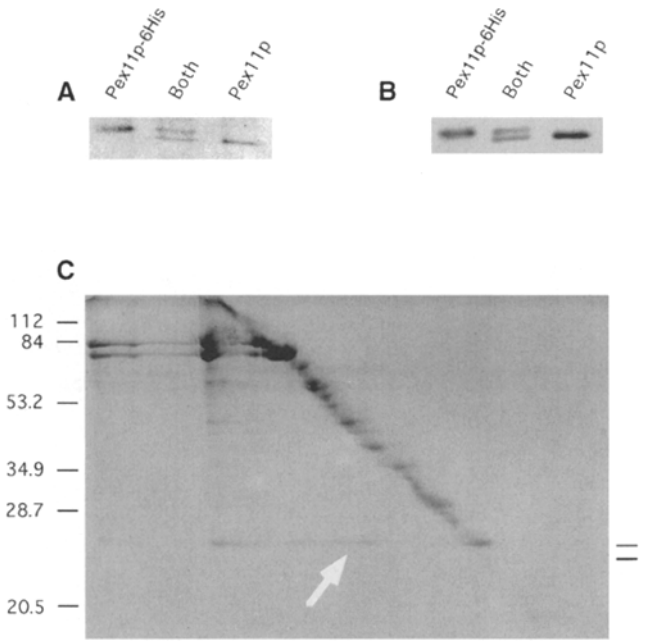

Figure 7. Pex11p forms homodimers. (*A*) Pex11p-6His can be resolved from wild-type Pex11p. Coomassie-stained gel of isolated peroxisomes showing resolution of the two protein species. (*B*) Immunoblotting of the same samples with the anti-Pex11p antibody showing resolution. (*C*) Peroxisomes from cells expressing only Pex11p-6His were cross-linked with DTSSP and were processed for two-dimensional electrophoresis as in Fig. 6 *A*. The arrow indicates Pex11p-6His migrating off of the diagonal. The two bars to the right of the figure indicate the migration on this gel of wild-type and Pex11p-6His (bottom and top bars, respectively).

age in the C3A mutant increases the monomer/dimer ratio and causes hyperproliferation, lending further support to this model.

This model also makes predictions from our previous work. The overexpression of Pex11p should initally cause an increase in monomers, thereby increasing peroxisomal fission to create an abundance of organelles. Also, the Pex11p introduced into the large peroxisomes of the Δ*pex11* strain, which transforms the large organelles into many more smaller organelles, should exist mainly as monomer at early time points. Future work will test these predictions.

A basic issue that remains unanswered is the mechanism of action of Pex11p in promoting fission. Our data suggest that its action is from within the organelles, a mechanism quite different from those thought to control the division of other organelles. For example, organelles of the secretory system use coat proteins that form on the cytosolic side of the membrane to facilitate their budding (reviewed in Rothman and Wieland, 1996, and Scheckman and Orci, 1996). These coat proteins are varied in their nature and include clathrin, COPI, and COPII. Clathrin concentrates receptors within the coated pit on the cell surface. Clathrin within the pit progressively rearranges to form a bud structure which eventually pinches off and is released into the cytosol. COPI and COPII are recruited from the cytosol to the endoplasmic reticulum or Golgi membranes by small G proteins, and uncoating is required before any of these

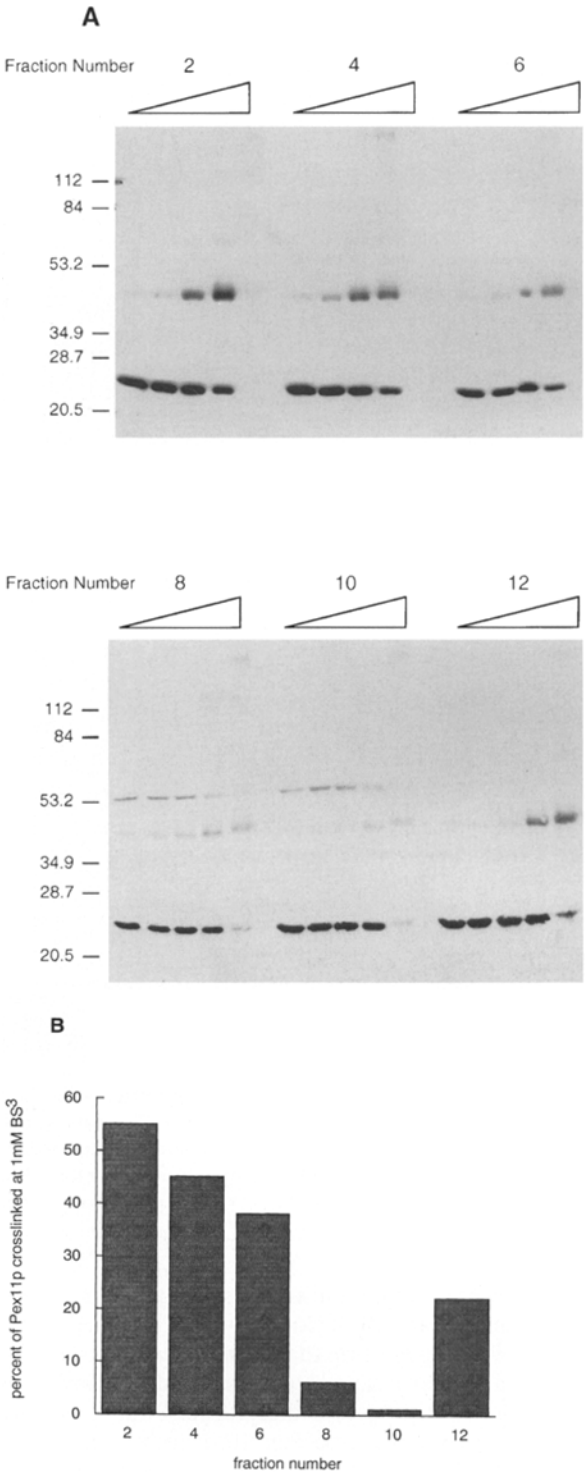

Figure 8. A higher percentage of Pex11p is cross-linked to dimer in mature peroxisomes. (*A*) Even-numbered Nycodenz gradient fractions were normalized for Pex11p concentration and protein concentration and were subjected to increasing concentrations of BS^3. Concentrations of cross-linker are the same as in Fig. 5. Two gels were used for this experiment, accounting for slight differences in absolute band intensity between upper and lower panel. (*B*) Graphical representation of (*A*) at 1 mM BS^3.

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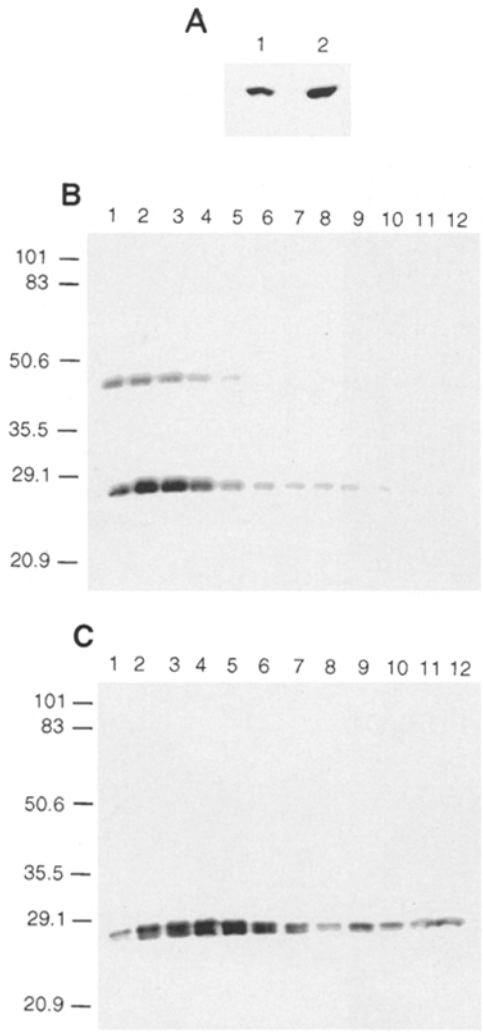

Figure 9. Pex11p forms homodimers on nonreducing SDS-PAGE, but the pex11p C3A mutant does not. (*A*) 40 μg of protein from whole cell lysates of cells grown 16 h in HMYO were subjected to SDS-PAGE and Western blotting with the anti-Pex11p antibody. (Lane *1*) Cells expressing the pex11p C3A mutant. (Lane *2*) Cells expressing wild-type Pex11p. (*B* and *C*) Nycodenz gradient fractions from cells expressing either wild-type Pex11p (*B*) or pex11p C3A (*C*) were subjected to nonreducing SDS-PAGE and immunoblotting with the anti-Pex11p antibody.

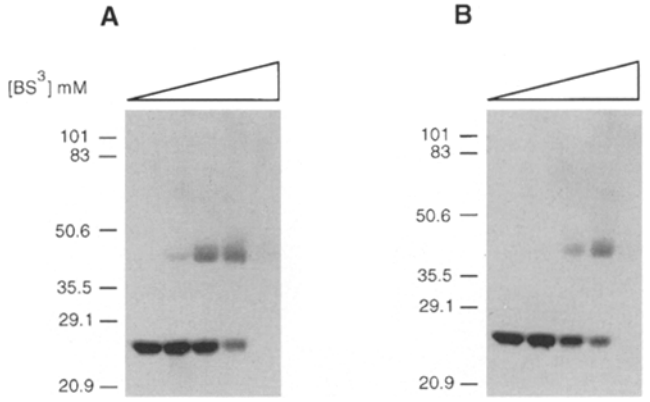

Figure 10. There is less cross-linked dimer in the C3A mutant than in wild type. Organellar pellets from the Δ*pex11* strain expressing wild-type Pex11p (*A*) or pex11p C3A (*B*) were subjected to increasing concentrations of the cross-linker BS^3. Concentrations of cross-linker are the same as shown in Fig. 5. Proteins were resolved by SDS-PAGE and Pex11p was detected by immunoblotting.

coated vesicles can fuse with their target membrane. These coats are necessary to deform the bilayer for vesicle budding. Transmembrane proteins such as p24 (Stamnes et al., 1995) are necessary to transduce a signal from the inside (luminal side) of the vesicle to the cytoplasmic side to instruct coatamer when to assemble. No such coat-mediated mechanism appears to function for peroxisomes, and no small G proteins (or trimeric ones) have been reported on highly purified peroxisomal membranes.

Mitochondrial division, however, may also depend on protein–protein interactions on the outer organelle surface. Mitochondria are closely apposed to the cytoskeleton and seem to rely on that cellular infrastructure for their shape and division (Bereiter-Hahn and Voth, 1994). The two proteins found to date that affect mitochondrial shape and segregation are Mdm10p (Sogo and Yaffe, 1994) and Mmm1p (Burgess et al., 1994), both from *S. cerevisiae.* Cells that have a mutation in either of these two proteins have large spherical mitochondria that have segregation defects. Both of these proteins are in the outer mitochondrial membrane and have been hypothesized to interact with the cytoskeleton through their cytoplasmic domains.

At this point no peroxisomal proteins have been found that interact with the cytoskeleton. Schrader et al. have shown, however, that peroxisomes can interact with the cytoskeleton in HepG2 cells, probably through a loosely associated factor from the cytosol (Schrader et al., 1996). Tubular, presumably budding, peroxisomes are not associated with the cytoskeleton; instead spherical organelles are associated. Thus, peroxisomes may rely on the cytoskeleton primarily for assistance in segregation during cell division (constitutive mechanism). Perhaps Pex11p has an indirect role in disassociating peroxisomes from the cytoskeleton, an action which may be essential for proliferation, whether constitutive or regulated. This might be tested by the use of actin-depolymerizing drugs or conditional actin mutants. It is still possible that Pex11p interacts substoicheometrically with an integral membrane protein that in turn communicates with the cytoskeleton.

Peroxisomal proliferation may be more related to the budding of certain viruses, another process that is driven from within. Vesicular stomatitis virus M protein seems to play a critical role in the budding of that virion. This protein, when expressed alone, promotes bud formation and release from the cell surface (Li et al., 1993), suggesting that this protein is driving the budding process. Fluorescence resonance energy transfer studies carried out with the M protein of vesicular stomatitis virus demonstrated that the M protein can induce formation of membrane domains either enriched in phosphatidic acid or phosphatidylserine (Luan et al., 1995; Luan, 1994). In vitro experiments seem to indicate that domain formation is probably the first step in membrane alterations that produce budding (Döbereiner et al., 1993; Lipowsky, 1993; Sackmann and Feder, 1995). Perhaps Pex11p also causes formation of

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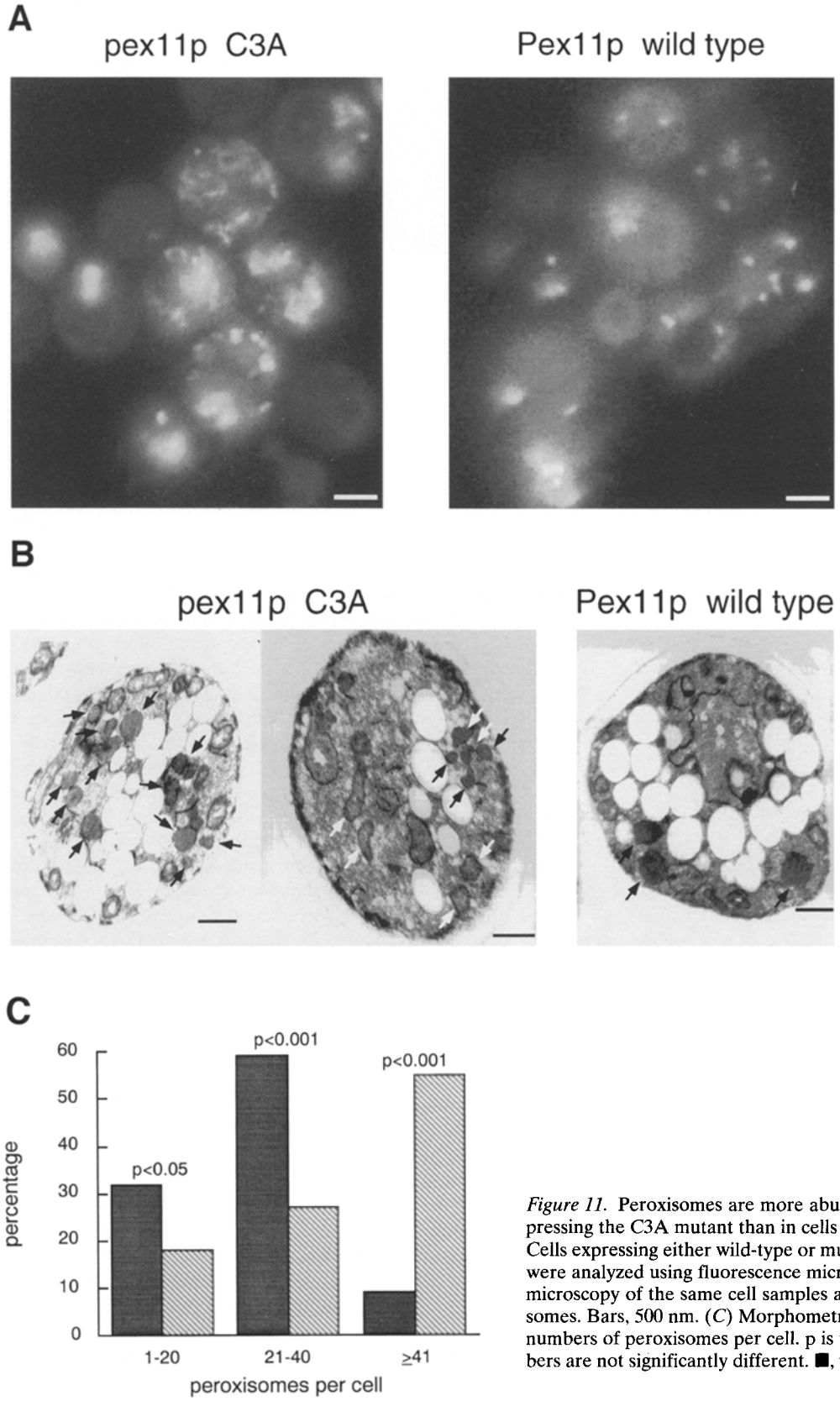

Figure 11. Peroxisomes are more abundant and smaller in the cells expressing the C3A mutant than in cells expressing wild-type Pex11p. (*A*) Cells expressing either wild-type or mutant C3A Pex11p and GFP-AKL were analyzed using fluorescence microscopy. Bars, 1 μm. (*B*) Electron microscopy of the same cell samples as in (*A*). Arrows indicate peroxisomes. Bars, 500 nm. (*C*) Morphometry on GFP-AKL–expressing cells: numbers of peroxisomes per cell. p is the probability that the two numbers are not significantly different. ■, wt Pex11p; ▨, Pex11p C3A.

lipid domains to induce peroxisomal budding. These Pex11p-induced lipid domains could cause the peroxisomes to dissociate from the cytoskeleton, thus promoting tubulation and budding. One difference in these systems is that the M protein is synthesized in the cytoplasm and interacts with the cytoplasmic face of the plasma membrane to affect budding (Pal and Wagner, 1987) while Pex11p must first cross the peroxisomal membrane to perform its

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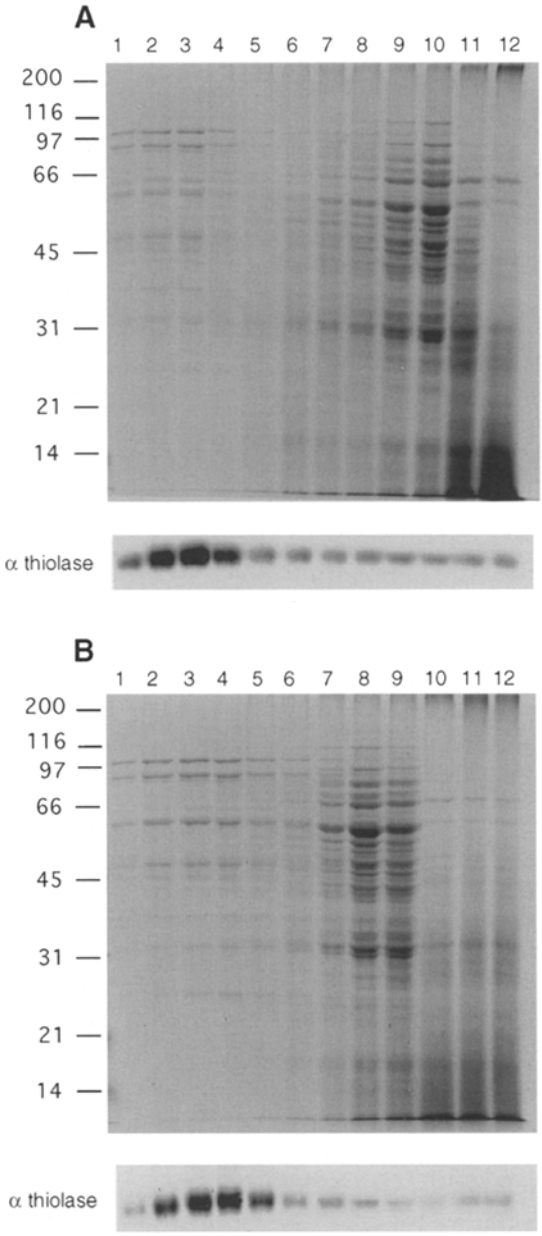

Figure 12. Peroxisomes in cells expressing the pex11p C3A mutant are less dense than peroxisomes from cells expressing wild-type Pex11p. (*A*) Coomassie-stained gel of Nycodenz fractions from cells expressing wild-type Pex11p and antithiolase immunoblot of the same fractions to indicate peroxisomal migration. (*B*) As in (*A*), but cells expressed pex11p C3A instead of the wild-type protein.

function on the matrix side. Pex11p might be prevented from interacting on the outer surface of peroxisomes by an active transport mechanism or it may not be able to perform its function until it is localized to the lipid environment of the inner peroxisomal leaflet.

Pex11p might also promote fission by perturbing or bending the membrane in a way predicted from the bilayer-couple hypothesis (Sheetz and Singer, 1974). Simply stated, this holds that two halves of a membrane bilayer can respond differently to disturbances but still be coupled to each other by contact forces. The two halves of the bilayer respond differently because they have different lipid and protein compositions. Thus peroxisomes with Pex11p in the inner leaflet may respond to perturbations in such a way as to cause or promote budding or fission. These Pex11p-induced alterations in membrane structure might also promote dissociation of the organelle from the cytoskeleton (Schrader et al., 1996). Without Pex11p, the two leaflets may respond more similarly and thus budding is inhibited.

But whether Pex11p interacts with the inner leaflet of the peroxisome to cause its expansion (simply by virtue of its presence in large concentration) or its contraction (by acting as a "flippase" or sequestering lipids with smaller unit surface area [Farge and Devaux, 1992; Käs and Sackmann, 1991]) to promote budding is unknown. Regardless of mechanism, we predict that this interaction with lipid is fundamentally altered by dimer formation. The amphipathic amino terminus of Pex11p (Marshall et al., 1995; Moreno et al., 1994) probably interacts directly with the membrane in a way that is essential for its function, and formation of the cystine crossbridge could draw this region out of the membrane and into a more proteinaceous environment.

Our cross-linking data (Fig. 6 *C*) suggest that there are higher order forms of Pex11p, since multiple regularly spaced spots appear by two-dimensional gel analysis. These correspond in size to tetramer, hexamer, and octamer. These data might be explained if the smallest functional unit of Pex11p that we term monomer is actually a dimer of Pex11p molecules that are noncovalently associated through hydrophobic interactions between their carboxy terminal domains. In this model, the amino-terminal domains (containing the cysteine residues) are active in initiating the proliferation process. Disulfide formation between the amino termini of adjacent Pex11p dimers would then terminate the proliferative function of the amino-terminal domains and result in the formation of higher order assemblies, which may serve as an infrastructure for peroxisomal architecture. Perhaps the particular cross-linkers that we used have a lower affinity for the hydrophobic domain interactions and a higher affinity for the disulfide-stabilized NH_2-terminal domains. This would explain the excellent correlation between the cross-linking data and the presence or absence of a disulfide bond. It would also explain the appearance of the higher order structures that are multiples of two in the cross-linking experiments (see Fig. 6 *C*). This model is fully consistent with the data presented in this paper because the nonreducing gels were run under denaturing conditions, which would disrupt hydrophobic interactions while preserving covalent interactions. It should be noted that Pex11p is the most abundant protein in high pH-stripped membranes (McCammon et al., 1990), so any other heterophilic interactions, if they occur, may involve matrix proteins or other peripheral elements. This hypothesis proposes that the smallest functional unit of Pex11p, regardless of peroxisomal status, is the dimer. We hope to test this idea, as well as study the mechanisms of Pex11p-induced budding and inactivation, with reconstituted systems.

If disulfide-linked Pex11p terminates division of mature organelles, how can new peroxisomes be generated? A possible solution is that new peroxisomes do not bud from

preexisting mature organelles, but from more immature progenitors. There is growing evidence of heterogeneity among peroxisomes, based on both morphological and biochemical criteria (Schrader et al., 1994; van Roermund et al., 1995; Wilcke et al., 1995; Yamamoto and Fahimi, 1987; Lüers et al., 1993). But there is also evidence that peroxisomes can bud from seemingly mature organelles (Veenhuis et al., 1978), and that most peroxisomes are competent for import (Hill and Walton, 1995). Although this issue is unresolved, our data predict that a subpopulation of peroxisomes, those with mainly dimeric Pex11p, are end-stage, while those capable of division have a high concentration of monomers. However, it is possible that an influx of newly synthesized monomeric Pex11p could initiate proliferation regardless of the previous Pex11p monomer/dimer ratio. Further work, perhaps in which the monomer/dimer ratio is artificially altered by changes in peroxisomal metabolism, might lead to a test of this prediction.

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