Pex30p, Pex31p, and Pex32p Form a Family of Peroxisomal Integral Membrane Proteins Regulating Peroxisome Size and Number in *Saccharomyces cerevisiae*

Franco J. Vizeacoumar,* Juan C. Torres-Guzman,** David Bouard,*
John D. Aitchison,† and Richard A. Rachubinski*§

*Department of Cell Biology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada; †Instituto de Investigaciones en Biología Experimental, University of Guanajuato, Guanajuato, Mexico; and ‡The Institute for Systems Biology, Seattle, Washington 98103

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The peroxin Pex23p of the yeast *Yarrowia lipolytica* exhibits high sequence similarity to the hypothetical proteins Ylr324p, Ygr004p, and Ybr168p encoded by the *Saccharomyces cerevisiae* genome. Ylr324p, Ygr004p, and Ybr168p are integral to the peroxisomal membrane and act to control peroxisome number and size. Synthesis of Ylr324p and Ybr168p, but not of Ygr004p, is induced during incubation of cells in oleic acid-containing medium, the metabolism of which requires intact peroxisomes. Cells deleted for *YLR324w* exhibit increased numbers of peroxisomes, whereas cells deleted for *YGR004w* or *YBR168w* exhibit enlarged peroxisomes. Ylr324p and Ybr168p cannot functionally substitute for one another or for Ygr004p, whereas Ygr004p shows partial functional redundancy with Ylr324p and Ybr168p. Ylr324p, Ygr004p, and Ybr168p interact within themselves and with Pex28p and Pex29p, which have been shown also to regulate peroxisome size and number. Systematic deletion of genes demonstrated that *PEX28* and *PEX29* function upstream of *YLR324w*, *YGR004w*, and *YBR168w* in the regulation of peroxisome proliferation. Our data suggest a role for Ylr324p, Ygr004p, and Ybr168p—now designated Pex30p, Pex31p, and Pex32p, respectively—together with Pex28p and Pex29p in controlling peroxisome size and proliferation in *Saccharomyces cerevisiae*.

INTRODUCTION

Peroxisomes are highly responsive organelles, because their size, number, protein composition and biochemical functions vary dramatically depending on the organism, cell type, and environmental milieu. Peroxisomes are essential for normal human development and physiology, as demonstrated by the lethality of the peroxisome biogenesis disorders, a group of autosomal recessive diseases in which multiple peroxisomal metabolic pathways are dysfunctional because peroxisome biogenesis is compromised. Peroxisome assembly, division, and inheritance are controlled by at least 29 proteins termed peroxins that are encoded by the *PEX* genes. To date, mutations in 12 *PEX* genes have been shown to give rise to the peroxisome biogenesis disorders (reviewed in Subramani *et al.*, 2000; Purdue and Lazarow, 2001; Titorenko and Rachubinski, 2001; Brosius and Gärtner, 2002; Matsumoto *et al.*, 2003).

Peroxisomal proteins are encoded exclusively by nuclear genes, synthesized on cytosolic polysomes and posttransla-

tionally targeted to the peroxisome. They are sorted to the peroxisome by specific peroxisome targeting signals (PTSs). PTSs are recognized in the cytosol by their cognate receptors, which direct the targeting and docking of proteins to the peroxisome membrane. Most peroxisomal matrix proteins are targeted by PTS1, an extreme carboxyl-terminal tripeptide, and its shuttling receptor, the peroxin Pex5p. Strikingly, Pex5p has been shown to enter the peroxisome matrix together with its cargo and to recycle back to the cytosol following dissociation from its cargo (Dammai and Subramani, 2001). A few matrix proteins are targeted by PTS2, an amino-terminal nonapeptide, and its cytosolic shuttling receptor, Pex7p. A limited number of matrix proteins are targeted by largely uncharacterized internal PTSs. The sorting of peroxisomal membrane proteins is much less understood and appears to be independent of matrix protein import. The cytosolic form of the peroxin Pex19p may act as a shuttling receptor for peroxisomal membrane proteins, whereas its peroxisome-associated form could function as a chaperone, assisting the assembly of multimeric complexes after their binding to the peroxisome membrane (reviewed in Subramani, 1998; Hettema *et al.*, 1999; Subramani *et al.*, 2000; Terlecky and Fransen, 2000; Purdue and Lazarow, 2001; Titorenko and Rachubinski, 2001).

In contrast to protein sorting to peroxisomes, much less is known about the mechanism of peroxisome proliferation and the proteins involved in this process. A few proteins have been implicated directly in regulating this process. Oversynthesis of Pex11p leads to the formation of small

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§ Corresponding author. E-mail address: rick.rachubinski@ualberta.ca.

Abbreviations used: 20KgP, 20,000 × g pellet; 20KgS, 20,000 × g supernatant; DsRed, red fluorescent protein from *Discosoma* sp.; ORE, oleic acid response element; ORF, open reading frame; PNS, postnuclear supernatant; PTS, peroxisome targeting signal.

Table 1. Yeast strains used in this study

Strain	Genotype	Derivation
BY4741	<i>MATα, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0</i>	Giaever <i>et al.</i> , 2002
BY4742	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0</i>	Giaever <i>et al.</i> , 2002
BY4743	<i>MATα/MATα, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, met15Δ0/+ , +/lys2Δ0, ura3Δ0/ura3Δ0</i>	Giaever <i>et al.</i> , 2002
DF5α	<i>MATα, ura3-52, his3-200, trp1-1, leu2-3,112,lys2-801</i>	Finley <i>et al.</i> , 1987
DF5α	<i>MATα, ura3-52, his3-200, trp1-1, leu2-3,112, lys2-801</i>	Finley <i>et al.</i> , 1987
SFY526	<i>MATα, ura3-52, his3-200, ade2-101, lys2-801Trp1-901, leu2-3,112,gal4-542, gal80-538,LYS::GALI_{UAS}-GAL1_{TATA}-lacZ,MEL1</i>	Harper <i>et al.</i> , 1993
PEX3-prA	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pex3::PEX3-protA (HIS5)</i>	Vizeacoumar <i>et al.</i> , 2003
PEX17-prA	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pex17::PEX17-protA (HIS5)</i>	Vizeacoumar <i>et al.</i> , 2003
POT1-prA	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1::POT1-protA (HIS5)</i>	This study
YLR324w-prA	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, Ylr324w::YLR324w-protA (HIS5)</i>	This study
YGR004w-prA	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, Ygr004w::YGR004w-protA (HIS5)</i>	This study
YBR168w-prA	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, ybr168w::YBR168w-protA (HIS5)</i>	This study
ylr324Δ	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, ylr324w::KanMX4</i>	Giaever <i>et al.</i> , 2002
ygr004Δ	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, ygr004w::KanMX4</i>	Giaever <i>et al.</i> , 2002
ybr168Δ	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, ybr168w::KanMX4</i>	Giaever <i>et al.</i> , 2002
DK1	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, ylr324w::KanMX4, ygr004w::KanMX4</i>	This study
DK2	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, ylr324w::KanMX4, ybr168w::KanMX4</i>	This study
DK3	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, ygr004w::KanMX4, ybr168w::KanMX4</i>	This study
CD1	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, pex28::KanMX4, ylr324w::KanMX4</i>	This study
CD2	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, pex28::KanMX4, ygr004w::KanMX4</i>	This study
CD3	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, pex28::KanMX4, ybr168w::KanMX4</i>	This study
CD4	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, pex29::KanMX4, ylr324w::KanMX4</i>	This study
CD5	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, pex29::KanMX4, ygr004w::KanMX4</i>	This study
CD6	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, pex29::KanMX4, ybr168w::KanMX4</i>	This study
TKO	<i>MATα, his3Δ1, leu2Δ0, ura3Δ0, ylr324w::KanMX4, ygr004w::KanMX4ybr168w::NAT</i>	This study
ybr168Δ-NAT	<i>MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, ybr168w::NAT</i>	This study

peroxisomes, whereas cells lacking Pex11p have fewer but larger peroxisomes than normal (Erdmann and Blobel, 1995; Marshall *et al.*, 1995; Sakai *et al.*, 1995; Li and Gould, 2002; Li *et al.*, 2002). The dynamin-like protein Vps1p (Hoepfner *et al.*, 2001) and the peroxins Pex25p (Smith *et al.*, 2002) and Pex27p (Tam *et al.*, 2003; Rottensteiner *et al.*, 2003b) of the yeast *Saccharomyces cerevisiae* have recently been shown to be required for the control of peroxisome size and number. Cells deleted for *VPS1*, *PEX25*, or *PEX27* contain enlarged peroxisomes.

YIPex23p is a 48-kDa integral membrane protein of peroxisomes required for peroxisome assembly in the yeast *Yarrowia lipolytica* (Brown *et al.*, 2000). A search of protein databases revealed that YIPex23p shares extensive sequence similarity with proteins encoded by the open reading frames (ORF) *YLR324w*, *YGR004w*, and *YBR168w* of the *S. cerevisiae* genome. Here we report that *YLR324w*, *YGR004w*, and *YBR168w* all encode peroxisomal integral membrane proteins, and we provide evidence for their role in controlling peroxisome size and number in *S. cerevisiae*.

MATERIALS AND METHODS

Strains and Culture Conditions

The yeast strains used in this study are listed in Table 1. All strains were cultured at 30°C. Media components were as follows: YPD, 1% yeast extract, 2% peptone, 2% glucose; YPBO, 0.3% yeast extract, 0.5% peptone, 0.5% K₂HPO₄, 0.5% KH₂PO₄, 1% Brij 35, 1% (vol/vol) oleic acid; synthetic minimal (SM) medium, 0.67% yeast nitrogen base without amino acids, 2% glucose, 1× Complete Supplement Mixture (Bio 101, Carlsbad, CA) without histidine and/or leucine; sporulation medium, 1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% agar; YNBD, 0.67% yeast nitrogen base without amino acids, 2% glucose, containing histidine, leucine, and uracil, each at 30 μg/ml.

Plasmids

The plasmids pDsRed-PTS1 (Smith *et al.*, 2002) and pProtA/HIS5 (Rout *et al.*, 2000) have been described. Genes to be overexpressed were amplified by PCR

and cloned into the plasmid YEpl3 (Broach *et al.*, 1979). For overexpression, the *YLR324w* gene included 609 base pairs of upstream and 348 base pairs of downstream sequence, the *YGR004w* gene included 625 base pairs of upstream and 217 base pairs of downstream sequence, and the *YBR168w* gene included 592 base pairs of upstream and 381 base pairs of downstream sequence. *PEX28* and *PEX29* overexpression constructs have been described (Vizeacoumar *et al.*, 2003).

Protein A-tagging of Candidate Proteins

Genes were genomically tagged with the sequence encoding *Staphylococcus aureus* protein A by homologous recombination using PCR-based integrative transformation into parental BY4742 haploid cells (Aitchison *et al.*, 1995; Dilworth *et al.*, 2001). The functionality of fusion proteins was confirmed by the ability of transformed strains to grow and to proliferate peroxisomes like the wild-type strain BY4742 in medium containing oleic acid as the sole carbon source.

Microscopy

Strains encoding protein A chimeras were transformed with the plasmid pDsRed-PTS1 were grown in SM medium for 12 h and then incubated in YPBO medium for 8 h. Cells were processed for immunofluorescence microscopy as described (Pringle *et al.*, 1991; Tam and Rachubinski, 2002). Protein A chimeras were detected with rabbit antiserum to mouse IgG (ICN Biomedicals, Irvine, CA) and FITC-conjugated goat anti-rabbit IgG. Images were captured on a LSM510 META (Carl Zeiss MicroImaging, Thornwood, NY) laser scanning microscope or with a digital fluorescence camera (Spot Diagnostic Instruments, Sterling Heights, MI). Whole cells were processed for electron microscopy as described (Eitzen *et al.*, 1997).

Morphometric Analysis of Peroxisomes

For each strain analyzed, electron microscopic images of 50 randomly selected cells at 17,000× magnification were captured with a digital camera (Soft Imaging System, Lakewood, CO), and the areas of individual cells and of individual peroxisomes were determined by the program analySIS 3.1 (Soft Imaging System). To determine the average area of a peroxisome, the total peroxisome area was calculated and divided by the total number of peroxisomes counted. To quantify peroxisome number, the numerical density of peroxisomes (number of peroxisomes per μm³ of cell volume) was calculated by the method of Weibel and Bolender (1973) for spherical organelles. First, the total number of peroxisome profiles was counted and reported as the number of peroxisomes per cell area assayed (*N_A*). Next, the peroxisome volume density (*V_V*) was calculated (total peroxisome area/total cell area

assayed). Using the values V_v and N_A , the numerical density of peroxisomes was determined (Weibel and Bolender, 1973).

Subcellular Fractionation and Isolation of Peroxisomes

Subcellular fractionation and isolation of peroxisomes were done essentially as described (Smith *et al.*, 2002; Vizeacoumar *et al.*, 2003). Briefly, cells grown overnight in YPD medium were transferred to YPBO medium and incubated for 8 h. Cells were harvested, washed, and converted to spheroplasts by digestion with Zymolyase 100T (1 mg/g of cells) in 50 mM potassium phosphate, pH 7.5, 1.2 M sorbitol, and 1 mM EDTA for 1 h at 30°C. Spheroplasts were lysed by homogenization in buffer H (0.6 M sorbitol, 2.5 mM MES, pH 5.5, 1 mM KCl) containing 1 mM EDTA and 2× complete protease inhibitor cocktail (Roche, Nutley, NJ). The homogenate was subjected to centrifugation for 10 min at 2000 × g to yield a postnuclear supernatant (PNS) fraction. The PNS fraction was subjected to further differential centrifugation at 20,000 × g for 30 min to yield a supernatant (20K_GS) fraction enriched for cytosol and a pellet (20K_GP) fraction enriched for peroxisomes. The 20K_GP fraction was resuspended in buffer H containing 11% Nycodenz and PINS (0.5 mM benzamide, 2 μg leupeptin/ml, 2 μg aprotinin/ml, 1 μg pepstatin A/ml, 3 μg antipain/ml, and 0.5 mg Pefabloc/ml), and a volume containing 5 mg of protein was overlaid onto a 30-ml discontinuous gradient consisting of 17, 25, 35, and 50% (wt/vol) Nycodenz in buffer H containing PINS. Organelles were separated by centrifugation at 100,000 × g for 90 min in a VT150 rotor (Beckman Coulter, Fullerton, CA). Fractions of 2 ml were collected from the bottom of the gradient.

Extraction of Peroxisomes

Peroxisomes were extracted as described (Fujiki *et al.*, 1982). Essentially, organelles in the 20K_GP fraction (50 μg of protein) were lysed by incubation in 10 volumes of Ti8 buffer (10 mM Tris-HCl, pH 8.0) containing 3× PINS on ice for 1 h and separated into pellet (Ti8P) and supernatant (Ti8S) fractions by centrifugation at 245,000 × g for 1 h at 4°C in a TLA120.2 rotor (Beckman Coulter). The Ti8P fraction was resuspended in Ti8 buffer to a final protein concentration of 0.5 mg/ml, and a portion of the resuspended fraction was extracted with 0.1 M Na₂CO₃, pH 11.3, for 1 h on ice and then separated into supernatant (CO₃S) and pellet (CO₃P) fractions by centrifugation at 245,000 × g in a TLA120.2 rotor at 4°C for 1 h. Proteins in the Ti8S, Ti8P, CO₃S, and CO₃P fractions were precipitated by addition of trichloroacetic acid, and the precipitates were washed with acetone. Proteins in equal portions of each fraction were separated by SDS-PAGE and analyzed by immunoblotting.

Construction of Haploid Strains Harboring Double Deletions of the YLR324w, YGR004w, YBR168w, PEX28, and PEX29 Genes

Strains harboring different double deletions of the YLR324w, YGR004w, YBR168w, PEX28, and PEX29 genes were constructed in the manner described below for a strain deleted for the YLR324w and YGR004w genes.

The homozygous deletion diploid strain ylr324Δ-HD (Gaever *et al.*, 2002) was sporulated, and the tetrads were dissected to select for the haploid MATα strain. This strain was mated to the haploid MATα deletion strain ygr004Δ by

Figure 1. Sequence alignment of *Yarrowia lipolytica* Pex23p with the proteins Ylr324p, Ygr004p, and Ybr168p encoded by the *S. cerevisiae* genome. Amino acid sequences were aligned with the use of the ClustalW program (EMBL-European Bioinformatics Institute, <http://www.ebi.ac.uk/clustalw/>). Identical residues in at least three of the proteins are shaded black, whereas identical residues in two proteins are shaded blue. Similar residues are shaded gray. Similarity rules: G = A = S; A = V; V = I = L = M; I = L = M = F = Y = W; K = R = H; D = E = Q = N; and S = T = Q = N.

replica plating to obtain a heterozygous diploid strain harboring deletions for both the *YLR324w* and *YGR004w* genes. The diploid strain was sporulated, and tetrads from 10 heterozygous diploids were dissected by micromanipulation. All spores were grown in YPD medium, and DNA was extracted. Haploid strains carrying deletions in both the *YLR324w* and *YGR004w* genes were confirmed by PCR analysis. In this way, nine strains containing the following double gene deletions were constructed: *ylr324Δ/ylr004Δ* (DK1), *ylr324Δ/ybr168Δ* (DK2), *ygr004Δ/ybr168Δ* (DK3), *pex28Δ/ylr324Δ* (CD1), *pex28Δ/ygr004Δ* (CD2), *pex28Δ/ybr168Δ* (CD3), *pex29Δ/ylr324Δ* (CD4), *pex29Δ/ygr004Δ* (CD5), and *pex29Δ/ybr168Δ* (CD6).

Construction of a Haploid Strain Harboring a Triple Deletion of the *YLR324w*, *YGR004w*, and *YBR168w* Genes

The gene conferring kanamycin resistance used to disrupt the *YBR168w* gene in the *MATa* strain of *ybr168Δ* was replaced with the gene encoding resistance to the drug nourseothricin (Werner BioAgents, Jena, Germany) from the plasmid pHN15 (Krügel *et al.*, 1993). This nourseothricin-resistant *MATa* strain deleted for *YBR168w* was crossed with the *MATa* strain DK1 (*YLR324w/YGR004w*), and the resultant diploid was sporulated. The strain TKO deleted for the *YBR168w*, *YLR324w*, and *YGR004w* genes was selected on agar plates containing both kanamycin and nourseothricin, and deletion of the three genes in this strain was confirmed by PCR analysis.

Two-hybrid Analysis

Physical interactions between Ylr324p, Ygr004p, Ybr168p, Pex28p, and Pex29p were detected using the Matchmaker Two-Hybrid System (Clontech, Palo Alto, CA). Chimeric genes were generated by amplifying the ORFs of the *YLR324w*, *YGR004w*, *YBR168w*, *PEX28*, and *PEX29* genes by PCR and ligating them in-frame and downstream of the DNA encoding the transcription-activating domain (AD) and the DNA-binding domain (DB) of the GAL4 transcriptional activator in the plasmids pGAD424 and pGBT9, respectively. Cells of *S. cerevisiae* strain SFY526 were transformed simultaneously with a pGAD424-derived plasmid and a pGBT9-derived plasmid. Transformants were grown on SM medium lacking tryptophan and leucine and tested for activation of the *lacZ* construct using a β -galactosidase filter assay (Smith and Rachubinski, 2001).

Antibodies

Antibodies to the carboxyl-terminal SKL tripeptide, thiolase, and Sdh2p have been described (Vizeacoumar *et al.*, 2003). FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-guinea pig IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used to detect primary antibodies in immunofluorescence microscopy. Rabbit antibodies to glucose-6-phosphate dehydrogenase (G6PDH) of *S. cerevisiae* were obtained from Sigma-Aldrich (St. Louis, MO).

Analytical Procedures

Extraction of nucleic acid from yeast lysates and manipulation of DNA were performed as described (Ausubel *et al.*, 1994). Immunoblotting was performed using a wet transfer system (Ausubel *et al.*, 1994), and antigen-antibody complexes in immunoblots were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Protein concentration was determined using a commercially available kit (Bio-Rad Laboratories, Richmond, CA) and bovine serum albumin as standard.

RESULTS

Ylr324p, *Ygr004p*, and *Ybr168p* Share Extensive Sequence Similarity with *Y. lipolytica* Pex23p

YIPex23p is an integral peroxisomal membrane protein that has been shown to be required for peroxisome assembly in the yeast *Y. lipolytica* (Brown *et al.*, 2000). A search of protein databases with the GENEINFO(R)BLAST Network Service of the National Center for Biotechnology Information revealed three proteins encoded by the ORFs *YLR324w*, *YGR004w*, and *YBR168w* of the *S. cerevisiae* genome that exhibit extensive sequence similarity to YIPex23p (Figure 1). YIPex23p and Ylr324p exhibit 36.3% amino acid identity and 20.9% amino acid similarity (at positions of nonidentity), YIPex23p and Ygr004p exhibit 35.6% amino acid identity and 19.3% amino acid similarity, and YIPex23p and Ybr168p exhibit 17.6% amino acid identity and 24.4% amino acid similarity, whereas Ylr324p, Ygr004p, and Ybr168p exhibit 9.3% amino acid identity and 20.7% amino acid similarity among themselves. Ylr324p is predicted to be a protein of

Figure 2. The levels of Ylr324p-prA and Ybr168p-prA, but not of Ygr004p-prA, are increased during incubation of *S. cerevisiae* in oleic acid-containing medium. Cells were grown for 16 h in glucose-containing YPD medium and then transferred to, and incubated in, oleic acid-containing YPBO medium. Aliquots of cells were removed from the YPBO medium at the times indicated, and total cell lysates were prepared. Equal amounts of protein from the total cell lysates were analyzed by SDS-PAGE and immunoblotting to visualize the protein A fusions and Pot1p. Antibodies directed against glucose-6-phosphatase (G6PDH) were used to confirm the loading of equal protein in each lane.

molecular weight 59,461 and to have two transmembrane helices at amino acids 3–22 and 191–209 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>; Krogh *et al.*, 2001). Ygr004p is predicted to be a protein of molecular weight 52,942 and to have four transmembrane helices at amino acids 93–111, 110–129, 178–195, and 226–244. Ybr168p is predicted to be a protein of molecular weight 48,578 and to have six transmembrane helices at amino acids 45–62, 69–86, 102–121, 178–197, 205–224, and 230–249. Partial functional redundancy among Ylr324p, Ygr004p, and Ybr168p (discussed below) may have prevented them from being identified as being involved in peroxisome biogenesis in *S. cerevisiae* by procedures involving random mutagenesis and negative selection for growth of yeast on oleic acid-containing medium.

Synthesis of Ylr324p and Ybr168p, But Not of Ygr004p, Is Induced during Incubation of Cells in Oleic Acid-containing Medium

The culturing of yeast cells in oleic acid-containing medium elicits a dual cellular response in that it promotes the proliferation of peroxisomes and induces the expression of many genes encoding peroxisomal proteins. Genomically encoded protein A chimeras of Ylr324p, Ygr004p, and Ybr168p were monitored to analyze the expression of *YLR324w*, *YGR004w*, and *YBR168w*, respectively, under the control of their endogenous gene promoters. Yeast strains

Table 2. Putative OREs in the promoter regions of the *YLR324w*, *YGR004w*, and *YBR168w* genes

Gene	ORE canonical sequence (CCGN ₃ TNAN _{8–12} CGG)
<i>PEX7</i>	CAGN ₁₀ TNAN ₃ CCG
<i>PEX13</i>	CGGN ₁₂ TNAN ₃ CCG
<i>YLR324w</i>	CGGN ₁₂ TNAN ₃ CCG
<i>YGR004w</i>	CGGN ₁₃ TNAN ₈ CCG
<i>YBR168w</i>	CAGN ₂ TNAN ₁₃ CCG

Figure 3. Ylr324p-prA, Ygr004p-prA, and Ybr168p-prA are primarily integral peroxisomal membrane proteins. (A) The subcellular distributions of protein A chimeras were compared with that of DsRed-PTS1 in oleic acid-incubated cells by double labeling, indirect immunofluorescence microscopy. Ylr324p-prA, Ygr004p-prA, and Ybr168p-prA, along with the peroxisomal matrix protein Pot1p, colocalize with DsRed-PTS1 in punctate structures characteristic of peroxisomes. Protein A chimeras were detected with rabbit antibodies to mouse IgG and FITC-conjugated goat anti-rabbit IgG secondary antibodies. (B) A PNS fraction was divided by centrifugation into a supernatant (20KgS) fraction enriched for cytosol and a pellet (20KgP) fraction enriched for peroxisomes. Equivalent portions of each fraction were analyzed. Immunoblotting with rabbit anti-Pot1p antibodies detected both the protein A chimeras shown and Pot1p. (C) Ylr324p-prA, Ygr004p-prA, and Ybr168p-prA cofractionate with peroxisomes. Organelles in the 20KgP fraction were separated by isopycnic centrifugation on a discontinuous Nycodenz gradient. Fractions were collected from the bottom of the gradient, and equal portions of each fraction were analyzed by immunoblotting. Fractions enriched for peroxisomes and mitochondria were identified by immunodetection of Pot1p and Sdh2p, respectively. (D) Peroxisomes purified by isopycnic density gradient centrifugation were lysed by treatment with 10 mM Tris-HCl, pH 8.0, releasing matrix proteins to a supernatant fraction (Ti8S) after centrifugation. The membrane-containing pellet fraction (Ti8P) was treated with 0.1 M Na₂CO₃, pH 11.3, and then subjected to centrifugation to yield a supernatant fraction (CO₃S) enriched for peripherally associated membrane proteins and a pellet fraction (CO₃P) enriched for integral membrane proteins. Equal portions of the respective supernatant and pellet fractions were analyzed by immunoblotting. Immunodetection of Pot1p, Pex17p-prA, and Pex3p-prA marked the fractionation profiles of a matrix, peripheral membrane and integral membrane protein, respectively.

synthesizing Ylr324p-prA, Ygr004p-prA, and Ybr168p-prA were grown in glucose-containing YPD medium and then shifted to oleic acid-containing YPBO medium. Aliquots of cells were removed at various times after the shift to YPBO medium, and their lysates were analyzed by SDS-PAGE and immunoblotting (Figure 2). Ylr324p-prA, Ygr004p-prA, and Ybr168p-prA were all detected in glucose-containing YPD medium at the time of transfer. The levels of Ylr324p-prA and Ybr168p-prA increased with time of incubation of cells in YPBO medium, but not as dramatically as the levels of Pot1p (peroxisomal thiolase). The levels of Ygr004p-prA did not show any apparent increase with time of incubation of cells in YPBO medium. It is noteworthy that the promoter regions of *YLR324w*, *YGR004w*, and *YBR168w* contain sequences that resemble the canonical sequence

CCGN₃TNAN₈₋₁₂CGG of the oleic acid response element (ORE; Table 2; Rottensteiner *et al.*, 2002; 2003a), which acts to increase gene transcription in *S. cerevisiae* in the presence of oleic acid as a carbon source through the binding of the transcription factors Pip2p and Oaf1p (Rottensteiner *et al.*, 1996; Karpichev *et al.*, 1997). Whether these sequences actually do function as OREs remains to be determined.

Ylr324p, *Ybr168p*, and *Ygr004p* Are Primarily Integral Membrane Proteins of Peroxisomes

A carboxyl-terminal PTS1 is sufficient to direct a reporter protein to peroxisomes. A fluorescent chimera between *Discosoma* sp. red fluorescent protein (DsRed) and the PTS1 Ser-Lys-Leu has been shown to target to peroxisomes of *S.*

cerevisiae (Wang *et al.*, 2001; Smith *et al.*, 2002). Genomically encoded protein A chimeras of Ylr324p, Ygr004p, and Ybr168p, and the peroxisomal matrix protein Pot1p were localized in oleic acid-induced cells by indirect immunofluorescence microscopy combined with direct fluorescence of DsRed-PTS1 to identify peroxisomes (Figure 3A). Ylr324p-prA, Ygr004p-prA, Ybr168p-prA, and Pot1p colocalized with DsRed-PTS1 to small punctate structures characteristic of peroxisomes by confocal microscopy.

Subcellular fractionation and organelle extraction were used to establish if Ylr324p, Ygr004p, and Ybr168p are associated with peroxisomes and to determine their suborganellar locations. Ylr324p-prA, Ygr004p-prA, and Ybr168p-prA, like Pot1p, preferentially localized to the 20Kp fraction enriched for peroxisomes (Figure 3B). Peroxisomes were isolated from the 20Kp fractions of each of the strains expressing Ylr324p-prA, Ygr004p-prA, or Ybr168p-prA. The gradients were fractionated, and equal portions of each fraction were analyzed by immunoblotting (Figure 3C). Ylr324p-prA, Ygr004p-prA, and Ybr168p-prA coenriched with the peroxisomal matrix protein thiolase (Pot1p) and not with the mitochondrial protein, Sdh2p. Therefore, both microscopic analysis and subcellular fractionation showed Ylr324p, Ygr004p, and Ybr168p to be peroxisomal proteins. Some amount of Ylr324p-prA was always present in the lighter fractions during the gradient isolation of peroxisomes, whereas Ygr004p-prA consistently enriched in fraction 9 at a density lighter than that of peroxisomes. Whether there is a selective liberation of a soluble form of Ylr324p-prA during the isolation of peroxisomes or there are vesicular elements containing either Ylr324p or Ygr004p remains to be determined. It is also noteworthy that Ygr004p-prA consistently migrated as two distinct molecular species in SDS-PAGE (see Figure 3C). The reason for this heterogeneity is unknown but could be due to some form of posttranslational modification, e.g., phosphorylation, of Ygr004p-prA.

Peroxisomes were hypotonically lysed by incubation in dilute alkali Tris buffer and subjected to centrifugation to yield a supernatant (Ti8S) enriched for matrix proteins and a pellet (Ti8P) enriched for membrane proteins (Figure 3D). The chimeras of Ylr324p, Ygr004p, and Ybr168p localized primarily to the Ti8P fraction, as did the chimeras of the peripheral peroxisomal membrane protein Pex17p (Huhse *et al.*, 1998) and the integral peroxisomal membrane protein Pex3p (Höfelfeld *et al.*, 1991). The soluble peroxisomal matrix protein Pot1p was found almost exclusively in the Ti8S fraction. The reproducible presence of some Ylr324p-prA in the Ti8S fraction may again be representative of a soluble form of this protein that is selectively liberated from peroxisomes during their isolation (see Figure 3, B and C). The Ti8P fractions were then extracted with alkali sodium carbonate and subjected to centrifugation (Figure 3D). This treatment releases proteins associated with, but not integral to, membranes (Fujiki *et al.*, 1982). Under these conditions, Ylr324p-prA, Ygr004p-prA, and Ybr168p-prA fractionated with Pex3p-prA to the pellet fraction enriched for integral membrane proteins, whereas Pex17p-prA fractionated to the supernatant fraction enriched for soluble proteins, including peripheral membrane proteins. These data suggest that Ylr324p, Ygr004p, and Ybr168p are primarily integral peroxisomal membrane proteins, as has been shown for Ylpex23p (Brown *et al.*, 2000).

Ylr324p, Ygr004p, and Ybr168p Act to Control the Number and Size of Peroxisomes

Immunofluorescence analysis of oleic acid-incubated wild-type BY4742 cells with antibodies to the carboxyl-terminal

Figure 4. Cells deleted for one or more of the *YLR324w*, *YGR004w* and *YBR168w* genes exhibit altered peroxisome morphology. Cells of the wild-type strain BY4742 and of the *ylr324Δ*, *ygr004Δ*, *ybr168Δ*, *ylr324wΔ/ygr004wΔ* (DK1), *ylr324wΔ/ybr168wΔ* (DK2), *ygr004wΔ/ybr168wΔ* (DK3), and *ylr324wΔ/ygr004wΔ/ybr168wΔ* (TKO) deletion strains were grown in YPD medium for 16 h, transferred to YPBO medium, and incubated for 8 h in YPBO medium. Cells were observed by immunofluorescence microscopy with antibodies to the PTS1 tripeptide SKL (SKL) or to the PTS2-containing protein, Pot1p. Rabbit primary antibodies (SKL) were detected with FITC-conjugated secondary antibodies. Guinea pig primary antibodies (Pot1p) were detected with rhodamine-conjugated secondary antibodies. Bar, 1 μ m.

PTS1 tripeptide Ser-Lys-Leu (SKL) or to the PTS2-containing enzyme Pot1p showed a pattern of small punctate structures characteristic of peroxisomes (Figure 4). In contrast, the majority of cells of the *ylr324Δ* strain showed increased numbers of punctate structures, whereas cells of the *ygr004Δ* and *ybr168Δ* strains showed a reduced number of often enlarged punctate structures. There was no evidence of increased cytosolic immunofluorescence with either anti-SKL or anti-Pot1p antibodies in cells deleted for one or more of the *YLR324w*, *YGR004w*, and *YBR168w* genes, suggesting that these genes do not encode proteins required for the import of matrix proteins into peroxisomes (Figure 4).

In electron micrographs, wild-type cells grown in oleic acid-containing medium contained characteristic peroxisomes 0.3 to 0.5 μ m in diameter that were well separated from one another (Figure 5, A and I). In contrast, cells of the *ylr324Δ* strain (Figure 5B) showed increased numbers of peroxisomes (Table 3) that were similar in size to peroxisomes of wild-type cells (Figure 5I; Table 3). Cells of the *ygr004Δ* (Figure 5C) and *ybr168Δ* (Figure 5D) strains exhibited similar numbers of peroxisomes to wild-type cells (Table 3), but these peroxisomes were noticeably

Figure 5. Cells harboring deletions in one or more of the *YLR324w*, *YGR004w* and *YBR168w* genes exhibit peroxisomes that are altered in number and/or size. Ultrastructure of wild-type BY4742 (A), *ylr324Δ* (B), *ygr004Δ* (C) *ybr168Δ* (D) *ylr324Δ/ygr004Δ* (E), *ylr324Δ/ybr168Δ* (F), *ygr004Δ/ybr168Δ* (G) and *ylr324Δ/ygr004Δ/ybr168Δ* (H) cells. Cells were grown in YPD medium for 16 h, transferred to YPBO medium and incubated in YPBO medium for 8 h. Cells were fixed and processed for electron microscopy. P, peroxisome; P*, peroxisome cluster. Bar, 1.0 μm . (I) Morphometric analysis of peroxisomes of oleic acid-incubated wild-type (WT) BY4742 and deletion mutant cells. Fifty randomly selected cells from each strain were analyzed by the program analySIS 3.1 (Soft Imaging System) to determine the areas of individual peroxisomes. Peroxisomes were then separated into size categories. A histogram was generated for each strain depicting the percentage of total peroxisomes occupied by the peroxisomes of each category. The numbers along the x-axis are the maximum sizes of peroxisomes in each category (in μm^2).

larger than wild-type peroxisomes, particularly in the case of *ybr168Δ* cells (Figure 5I; Table 3). Cells of strain *DK1* (Figure 5E) carrying deletions in the *YLR324w* and *YGR004w* genes exhibited a mixed phenotype of increased numbers of peroxisomes (Table 3) of normal to enlarged size (Figure 5I; Table 3). Cells of strain *DK2* (Figure 5F) carrying deletions in the *YLR324w* and *YBR168w* genes showed increased numbers of peroxisomes (Table 3) of normal to enlarged size (Figure 5I), some of which exhib-

ited clustering, whereas cells of strain *DK3* (Figure 5G) deleted for the *YGR004w* and *YBR168w* genes also contained greatly enlarged peroxisomes (Figure 5I; Table 3). Cells of the strain *TKO* carrying deletions in all three genes showed an approximately fivefold increase in the average number of peroxisomes per cell, but the size distribution of peroxisomes was similar to that of wild-type cells (Figure 5I). Our results suggest that Ylr324p acts primarily as a negative regulator of peroxisome number,

Table 3. Average area and numerical density of peroxisomes in cells of wild-type and mutant strains

Strain	Cell area assayed (μm^2)	Average area of peroxisomes ^a (μm^2)	Average number of peroxisomes per cell	Peroxisome count ^b	Numerical density of peroxisomes ^c
WT (BY4742)	402	0.06	1.32	0.16	0.73
<i>ylr324Δ</i>	447	0.05	3.02	0.34	1.75
<i>ygr004Δ</i>	434	0.09	1.68	0.19	0.70
<i>ybr168Δ</i>	461	0.13	1.72	0.19	0.56
<i>DK1</i>	478	0.07	2.20	0.23	0.95
<i>DK2</i>	490	0.08	2.64	0.27	1.02
<i>DK3</i>	463	0.12	1.98	0.21	0.66
<i>TKO</i>	498	0.08	6.24	0.63	2.43

^a Average area on micrographs.^b Number of peroxisomes counted per μm^2 of cell area on micrographs.^c Number of peroxisomes per μm^3 of cell volume (Weibel and Bolender, 1973).

whereas Ygr004p and particularly Ybr168p act as negative regulators of peroxisome size.

Overexpression of YGR004w Can Partially Complement the Abnormal Peroxisomal Morphology Observed in Cells Deleted for One or Both of YLR324w and YBR168w

Because cells deleted for one or more of the *YLR324w*, *YGR004w*, and *YBR168w* genes are compromised in their regulation of peroxisome size and number, we investigated the effects of overexpression of these genes on the overall peroxisome phenotype in cells harboring various combinations of deletions of these genes. Overexpression of *YLR324w*, *YGR004w*, and *YBR168w* (Figure 6A) in their respective deletion backgrounds led to restoration of the wild-type peroxisomal phenotype. Overexpression of *YLR324w* in cells deleted for one or both of *YGR004w* and *YBR168w* did not lead to the complementation of the peroxisomal phenotype observed in cells deleted for either or both of *YGR004w* and *YBR168w* (Figure 6B). Similarly, overexpression of *YBR168w* in cells deleted for one or both *YLR324w* and *YGR004w* did not lead to complementation of the peroxisomal phenotype seen in cells deleted for either or both of the *YLR324w* and *YGR004w* genes (Figure 6C). In contrast, overexpression of the *YGR004w* gene in cells deleted for one or both of the *YLR324w* and *YBR168w* gene led essentially to the restoration of the wild-type peroxisomal phenotype, although evidence of peroxisomal clustering was still observed in cells deleted for both the *YLR324w* and *YBR168w* genes (Figure 6, D and E). Taken together, these data suggest that Ylr324p and Ybr168p cannot functionally substitute for one another or for Ygr004p, whereas Ygr004p shows partial, but not complete, functional redundancy with Ylr324p and Ybr168p.

Interacting Partners of Ylr324p, Ygr004p, and Ybr168p

A limited yeast two-hybrid screen was performed to identify physical interactions between Ylr324p, Ygr004p, and Ybr168p and between these proteins and Pex11p, Pex25p, Pex28p, Pex29p, and Vps1p, which have also been implicated in the control of peroxisome size and number (Erdmann and Blobel, 1995; Marshall *et al.*, 1995; Hoepfner *et al.*, 2001; Smith *et al.*, 2002; Vizeacoumar *et al.*, 2003). Others have used this methodology to detect interactions between peroxins (for examples, see Girzalsky *et al.*, 1999; Smith and Rachubinski, 2001; Sichting *et al.*, 2003). Chimeric genes were made by ligating the ORFs of *YLR324w*, *YGR004w*,

YBR168w, *PEX11*, *PEX25*, *PEX28*, *PEX29*, and *VPS1* in-frame and downstream of sequences encoding one of the two functional domains (AD or DB) of the GAL4 transcriptional activator. All possible combinations of plasmid pairs encoding AD and DB fusion proteins were transformed into *S. cerevisiae* strain SFY526, and β -galactosidase filter detection assays were performed. Interactions were detected between Ylr324p and Ygr004p, Pex29p, and itself (Figure 7). Ygr004p also interacted with itself, whereas weak interactions were detected between Ybr168p and Ylr324p and between Ybr168p and Pex28p (Figure 7). No interactions were detected between Ylr324p, Ygr004p, or Ybr168p and any of Pex11p, Pex25p, Pex29p, and Vps1p (unpublished data).

PEX28 and PEX29 Function Upstream of YLR324w, YGR004w, and YBR168w

Because yeast two-hybrid analysis provided evidence of interaction between the *S. cerevisiae* homologues of YIPex23p and the *S. cerevisiae* homologues of YIPex24p and because cells of deletion mutants of the genes encoding these proteins are affected in the control of peroxisome size and number (data herein and Vizeacoumar *et al.*, 2003), we investigated the hierarchical organization of these genes. Strains containing systematic pairwise gene deletions of the *S. cerevisiae* homologues of YIPEX23 and YIPEX24 were made, namely *pex28Δ/ylr324Δ* (CD1), *pex28Δ/ygr004Δ* (CD2), *pex28Δ/ybr168Δ* (CD3), *pex29Δ/ylr324Δ* (CD4), *pex29Δ/ygr004Δ* (CD5), and *pex29Δ/ybr168Δ* (CD6). Electron microscopy analysis (Figure 8) showed that the majority of cells containing double deletions of a YIPEX23 gene homolog and a YIPEX24 gene homolog exhibited the clustered peroxisomal phenotype typical of *pex28Δ* and *pex29Δ* cells (Vizeacoumar *et al.*, 2003), although cells of the CD2 and CD3 strains (Figure 8, B and C, respectively) often exhibited enlarged peroxisomes that were well separated one from another. Together our data suggest that *YLR324w*, *YGR004w*, and *YBR168w* function downstream of *PEX28* and *PEX29*.

DISCUSSION

Studies combining electron microscopy morphometry, pulse-chase analysis of peroxisomal protein trafficking in vivo, and the isolation and protein characterization of distinct peroxisomal subforms have shown that yeast peroxisomes do not grow and divide at the same time (Veenhuis and Goodman, 1990; Tan *et al.*, 1995; Titorenko *et al.*, 2000).

Figure 6. Ultrastructure of cells overexpressing *YLR324w*, *YGR004w*, or *YBR168w*. Cells harboring deletions in one or more of the *YLR324w*, *YGR004w*, and *YBR168w* genes and overexpressing one of these three genes were grown in SM medium overnight, transferred to YPBO medium, and incubated in YPBO medium for 8 h. (A) *ybr168Δ* cells overexpressing *YBR168w* and exhibiting wild-type peroxisomal morphology. (B) *ybr324Δ/ygr004Δ* (strain *DK1*) cells overexpressing *YLR324w*. Note enlarged peroxisomes typical of *ygr004Δ* cells. (C) *ybr168Δ/ygr004Δ* (strain *DK3*) cells overexpressing *YBR168w*. Note inability of *YBR168w* overexpression to complement the enlarged peroxisome phenotype of *ygr004Δ* cells. *YGR004w* overexpression in cells deleted for (D) *YLR324w* and *YBR168w* (strain *DK2*) or for (E) the three genes *YLR324w*, *YBR168w*, and *YGR004w* (strain *TKO*) essentially restores wild-type peroxisome morphology but with some evidence of peroxisome clustering. P, peroxisome; P*, peroxisome cluster. Bar, 1 μ m.

There appears to be at least two different temporal patterns of peroxisome growth and division. In the yeast *Candida boidinii* (Veenhuis and Goodman, 1990), an initial event in peroxisome development is the extensive proliferation of immature peroxisomal vesicles containing only minor amounts of matrix proteins. This large increase in the number of immature peroxisomes by division precedes their growth through the import of membrane and matrix proteins and their conversion to mature organelles containing the complete set of peroxisomal proteins (Veenhuis and Goodman, 1990). The timing of events of peroxisome growth and division is different in *Y. lipolytica*. In this organism, the growth of immature peroxisomal vesicles, which is accomplished by the import of matrix proteins, and their development into mature peroxisomes occur before completely assembled mature peroxisomes undergo division (Titorenko *et al.*, 2000). Similar temporal patterns of peroxisome growth and division have been observed for the yeast *Hansenula polymorpha* (Tan *et al.*, 1995). In human cells, both immature

peroxisomal vesicles and mature peroxisomes are proposed to be able to divide (Gould and Valle, 2000). However, the division of immature peroxisomes before their growth and maturation by peroxisomal protein import can be seen only in some peroxin-deficient fibroblasts following reactivation or reexpression of an originally defective peroxin-encoding gene (Matsuzono *et al.*, 1999; South and Gould, 1999; Sacksteder and Gould, 2000). In normal human cells, in contrast, conversion of immature peroxisomal vesicles to mature peroxisomes by membrane and matrix protein import may occur before peroxisomes undergo division (Gould and Valle, 2000).

Members of the Pex11 family of peroxins, including Pex25p (Smith *et al.*, 2002) and Pex27p (Tam *et al.*, 2003; Rottensteiner *et al.*, 2003b) of *S. cerevisiae*, have been shown to effect peroxisome division in different organisms (Erdmann and Blobel, 1995; Marshall *et al.*, 1995; Sakai *et al.*, 1995; Li and Gould, 2002; Li *et al.*, 2002). The dynamin-like protein Vps1p has also been implicated in this process

Figure 7. Yeast two-hybrid analysis of the interaction partners of Ylr324p, Ygr004p, and Ybr168p. A β -galactosidase filter detection assay is presented. *SFY526* cells synthesizing both Gal4-AD (left columns) and Gal4-DB (right columns) fusion proteins were tested for β -galactosidase activity. The color intensities of transformants for each strain is shown.

(Hoepfner *et al.*, 2001), and we recently showed that the peroxisomal integral membrane proteins Pex28p and Pex29p are also involved in controlling the number, size, and separation of peroxisomes in *S. cerevisiae* (Vizeacoumar *et al.*, 2003). Here, we have identified three novel peroxisomal proteins encoded by the ORFs *YLR324w*, *YGR004w*, and *YBR168w* of *S. cerevisiae* and demonstrated that these proteins also act to control peroxisome size and number in this organism.

The identification of novel proteins required for peroxisome biogenesis in *S. cerevisiae* through their sequence similarity with known peroxins in other organisms enabled the identification of Pex28p and Pex29p (Vizeacoumar *et al.*, 2003). *YIPex23p* is a peroxisomal integral membrane protein required for peroxisome assembly in *Y. lipolytica* that shares extensive sequence similarity to three proteins of unknown function and unknown localization encoded by the ORFs *YLR324w*, *YGR004w*, and *YBR168w* of the *S. cerevisiae* genome. Genomically encoded protein A chimeras of Ylr324p, Ygr004p, and Ybr168p were shown by a combination of confocal microscopy and subcellular fractionation to be peroxisomal proteins. In their response to extraction by different chaotropic agents, Ylr324p, Ygr004p, and Ybr168p act primarily as integral membrane proteins.

Ylr324p, Ygr004p, and Ybr168p are not required for peroxisome assembly per se, as cells harboring deletions for one, two, or all three of these genes still contain peroxisomes that are unaffected in their capacity to import PTS1- or PTS2-containing proteins. These peroxisomes are functional, at least to a degree, because the cells harboring deletions of these genes are able to grow in oleic acid-containing medium with essentially the same kinetics as wild-type cells (unpublished data). *YLR324w*, *YGR004w*, and *YBR168w* are also apparently not required for peroxisome inheritance, because all cells deleted for one or more of these genes still

contained peroxisomes after numerous cell divisions. Also, if *YLR324w*, *YGR004w*, and *YBR168w* had a direct role in the inheritance of peroxisomes, one might expect that a loss of peroxisomes from cells over time resulting from the impaired segregation of peroxisomes into daughter cells would lead to inhibited growth in oleic acid-containing medium for the deletion strains as compared with the wild-type strain, which was not observed.

Peroxisomes in cells deleted for the *YLR324w*, *YGR004w*, and *YBR168w* genes are not normal and show distinctive phenotypic differences from wild-type peroxisomes. *ylr324Δ* cells showed increased numbers of peroxisomes versus wild-type cells, whereas *ygr004Δ* and *ybr168Δ* cells contained not only greater numbers of peroxisomes but also enlarged peroxisomes (Figure 5). Cells deleted for two of the *YLR324w*, *YGR004w*, and *YBR168w* genes contained increased numbers of generally enlarged peroxisomes. Cells of the strain deleted for all three genes contained increased numbers of smaller to normally sized peroxisomes. Morphometric analyses and quantification revealed a fivefold increase in the numbers of peroxisomes on average per cell for the triple deletion strain than for the wild-type strain. Although an occasional enlarged peroxisome was evident in cells deleted for all three genes, the peroxisomal phenotype of these cells strongly resembled that of cells deleted for only the *YLR324w* gene. The characteristics of peroxisomes of cells of the deletion strains are consistent with a role for *YLR324w*, *YGR004w*, and *YBR168w* in the control of peroxisome size and number within *S. cerevisiae* cells. Our results suggest that Ylr324p acts primarily acts as a negative regulator of peroxisome number, whereas Ygr004p and particularly Ybr168p act as negative regulators of peroxisome size. Nevertheless, Ygr004p shares some redundancy of function with Ylr324p and Ybr168p, but not vice versa, as overexpression of *YGR004w* in cells deleted for one or both of the *YLR324w* and *YBR168w* genes results essentially in the reestablishment of the wild-type peroxisome phenotype, but in contrast to wild-type cells, there is some evidence of peroxisome clustering. The reason why peroxisomes cluster in these overexpressing cells is unknown.

It is interesting to note that *Y. lipolytica* cells deleted for the *PEX23* gene also show evidence of abnormal peroxisomal divisional control. These cells lack mature peroxisomes but do accumulate small vesicular structures that contain both peroxisomal matrix and membrane proteins (Brown *et al.*, 2000). However, these membrane structures do not function as peroxisomes, because *pex23Δ* cells cannot grow on medium containing oleic acid as the sole carbon source. Therefore, although *YIPex23p*, like Ylr324p, Ygr004p, and Ybr168p, likely has a role in the regulation of peroxisome division, *YIPex23p* probably does not function identically to Ylr324p, Ygr004p, or Ybr168p in this process.

Pex28p and Pex29p have been implicated in the control of peroxisome size and number (Vizeacoumar *et al.*, 2003). A limited yeast two-hybrid screen revealed physical interactions among Ylr324p, Ygr004p, Ybr168p, Pex28p, and Pex29p. No interactions were detected between these five proteins and Pex11p, Pex25p, and Vps1p, which also have been shown to play a role in the control of peroxisome size and division. Further experimentation is required to determine whether these interactions are direct or bridged by other proteins. It is interesting to note that Ylr324p was shown to interact with Pex29p. Some amount of Ylr324p (Figure 3) and of Pex29p (Vizeacoumar *et al.*, 2003) was

Figure 8. Peroxisome morphology in *S. cerevisiae* cells deleted for a *YIPEX23* homolog and a *YIPEX24* homolog. Ultrastructure of *pex28Δ/ylr324Δ* (A), *pex28Δ/ygr004Δ* (B), *pex28Δ/ybr168Δ* (C), *pex29Δ/ylr324Δ* (D), *pex29Δ/ygr004Δ* (E), and *pex29Δ/ybr168Δ* (F) cells. Cells were grown in YPD medium for 16 h, transferred to YPBO medium and incubated in YPBO medium for 8 h. Cells were fixed and processed for electron microscopy. P, peroxisome; P*, peroxisome cluster. Bar, 1 μm.

always present in the 20Ks fraction in differential fractionation and in the less dense fractions during the gradient isolation of peroxisomes. Whether some portion of these proteins forms a complex and is localized to some compartment other than peroxisomes remains to be determined.

How might Ylr324p, Ygr004p, Ybr168p, Pex28p, and Pex29p act to control the abundance, size, and distribution of peroxisomes in the *S. cerevisiae* cell? Cells systematically deleted for one of the *YLR324w*, *YGR004w*, and *YBR168w* genes and one of the *PEX28* and *PEX29* genes exhibited clusters of peroxisomes typically observed in cells deleted for the *PEX28* or *PEX29* gene (Vizeacoumar *et al.*, 2003). Our data suggest that Pex28p and Pex29p act upstream of

Ylr324p, Ygr004p, and Ybr168p in controlling peroxisome abundance and size.

Organelles are highly dynamic structures that undergo fission and fusion to control their numbers and modify their morphology in response to intracellular and extracellular cues and to permit their correct segregation at cell division. As a consequence, the maintenance of compartmental integrity by the eukaryotic cell requires the tight coordination of mechanisms controlling these events. Many proteins, including those encoded by the genes *YLR324w*, *YGR004w*, and *YBR168w* of *S. cerevisiae*, are involved in controlling peroxisome number and size in the cell. Because of their role in the control of peroxisome size and number, we propose that *YLR324w*, *YGR004w*,

and *YBR168w* be designated as *PEX30*, *PEX31*, and *PEX32*, respectively, and their encoded peroxins as Pex30p, Pex31p, and Pex32p. The challenge remains to understand how the increasing number of proteins shown to be involved in controlling peroxisome number and size interplay among themselves and signal to the cell how to control its peroxisome dynamics.

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