

The Effect of Culture Medium Composition on the Localization of Peroxisomal
Membrane Proteins in Yeast

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Abstract

Formation and degradation of peroxisomes are not well understood, but their absence leads to serious consequences. In the study described in this thesis, I sought to (1) verify previous work indicating that switching from glucose-rich medium to lipid-rich medium induces peroxisome formation; (2) reveal that switching back from lipid-rich to glucose-rich medium induces peroxisome degradation; (3) test the hypothesis that Pex3p is a class II PMP that enters the peroxisome from the endoplasmic reticulum during *de novo* peroxisome formation; and (4) test the hypothesis that Pxa1p is a class I PMP that enters the peroxisomal membrane from the cytosol. I followed the pattern of fluorescence in yeast cells expressing Pex3p-Green Fluorescent Protein (GFP) and Pxa1p-GFP fusion proteins after cells were transferred either from a glucose-rich to a lipid-rich medium or vice versa. I succeeded in producing GFP transformants for two peroxisome genes. I also confirmed that switching to lipid-rich medium induced peroxisome formation and demonstrated that switching the peroxisomes back into the glucose medium triggered a rapid degradation. Evidence consistent with the hypotheses that Pex3p moves from the ER to the peroxisomal membrane and that Pxa1p is transported from the cytosol to the membrane was obtained, but the overall support was weak.

Introduction

Peroxisomes, organelles found within all eukaryotic cells, contain oxidizing enzymes that function in the metabolism of lipids, catabolism of nitrogen bases, and synthesis of plasmalogens and cholesterol (Monastyrska and Klionsky, 2006). The absence or malfunctioning of these organelles can lead to serious disorders in humans, such as Zellweger Syndrome, Infantile Refsum Disease, and Neonatal Adrenoleukodystrophy (Small *et al.*, 1988). However, though the peroxisomes play an important role in eukaryotic cells, the process of their formation and degradation is not well understood.

There are two main types of peroxisomal proteins: those that function in the peroxisomal matrix (or lumen) and those that function in the peroxisomal membrane. Proteins are targeted to the peroxisomal matrix by either peroxisome targeting signal 1 (PTS1) or 2 (PTS2) (Bascom *et al.*, 2003). PTS1 is the more common targeting signal and is a carboxyl-terminal tripeptide with the Ser-Lys-Leu sequence; PTS2 is an occasionally cleaved, amino-terminal nonapeptide (Bascom *et al.*, 2003). Membrane proteins use different targeting signals. According to Heiland and Erdmann (2005), the targeting sequences for the peroxisomal membrane proteins (PMPs) contain a basic amino acid sequence that is in combination with one or more transmembrane sequences. There are two pathways that allow the peroxisome membrane proteins to reach the peroxisome. Class I PMPs are posttranslationally imported from the cytosol into the peroxisomal membrane. Their import requires the peroxisomal proteins, Pex3p and Pex19p. Class II PMPs appear to follow two pathways, depending on whether the PMPs are being inserted into preexisting or newly formed peroxisomes. In the latter case, the

PMPs appear to be first incorporated into the endoplasmic reticulum (ER) and then are transported to the peroxisome (Hoepfner et al., 2005).

The budding yeast, *Saccharomyces cerevisiae*, has served as a model system for the study of peroxisome formation and degradation. Formation can be induced by transferring cells from a medium containing glucose as the primary energy source to a medium containing lipid as the primary source of energy. The yeast genome has been completely sequenced and can be manipulated relatively easily, making it possible to modify genes of interest.

My research focused on two peroxisomal membrane proteins, peroxisomal biogenesis factor 3 protein (Pex3p) and peroxisomal ABC transporter 1 protein (Pxa1p). Pxa1p functions in the peroxisomal membrane as part of an ABC (ATP-binding cassette) transporter (Shani *et al.*, 1995). It is homologous to the human adrenoleukodystrophy protein (Shani *et al.*, 1995). Pxa1p is oriented with the C-terminus on the cytosolic side of the peroxisomal membrane, which allows a label to be attached to the C-terminus without impairing the ability of the protein to be transported into the membrane (Shani *et al.*, 1995). Pex3p is a peroxin, a peroxisomal membrane protein which is needed for the import of proteins into the peroxisomal matrix and the insertion of class I proteins into the peroxisomal membrane (Bascom *et al.*, 2003). Pex3p is a class II PMP that enters the peroxisome from the ER during *de novo* peroxisome formation (Hoepfner et al., 2005). It is targeted to the membrane by an N-terminal 46 amino acid membrane signal (Bascom *et al.*, 2003). This allows for the protein to be tagged on its C-terminus without interfering with insertion.

In the study described in this thesis, I sought to (1) confirm previous work indicating that switching from glucose-rich medium to lipid-rich medium induces peroxisome formation; (2) demonstrate that switching back from lipid-rich to glucose-rich medium induces peroxisome degradation; (3) test the hypothesis that Pex3p is a class II PMP that enters the peroxisome from the endoplasmic reticulum during *de novo* peroxisome formation; and (4) test the hypothesis that Pxa1p is a class I PMP that enters the peroxisomal membrane from the cytosol. I followed the pattern of fluorescence in yeast cells expressing Pex3p-Green Fluorescent Protein (GFP) and Pxa1p-GFP fusion proteins after cells were transferred either from a glucose-rich to a lipid-rich medium or vice versa. I expected that, after transfer to lipid-rich medium, fluorescence in cells synthesizing Pex3p-GFP will initially be concentrated in strands and will later merge into sphere-like structures. I predicted that that fluorescence in Pxa1p-GFP cells will initially be diffuse and will later be concentrated in spherical structures. After transfer back into glucose-rich medium, I expected fluorescence in cells producing both Pxa1p-GFP and Pex3p-GFP to diminish as the peroxisomes are broken down in vacuoles.

Background

Transformation using short flanking homology-PCR fragments

This method can be used to insert one or more genes useful for labeling and/or screening into the gene of another organism (for example, genes for GFP (jellyfish) and kanamycin resistance (bacteria) can be inserted into a yeast gene). The genes to be inserted are flanked at both ends with short (~40 nt) sequences that are homologous to regions on both sides of the insertion site. Once the SFH-PCR fragment is in the nucleus of the target cell it will be inserted by homologous recombination (Fig. 1).

Target = 3' end of coding region of the gene (stop codon in italics)

AGGTCCCTGGACT*GACGATTACGCCTA*

SFH-PCR fragment

AGGTCCCTGGAC--gene for GFP--gene for kanamycin resistance--CGATTACGCCTA

Figure 1. Example of transformation using short flanking homology-PCR fragments. The target is the 3' end of the coding region of the gene (stop codon in italics). The stop codon will be replaced with the genes for GFP and kanamycin resistance.

Verification of correct insertion of SFH-PCR fragment using PCR

The correct insertion of the SHF-PCR fragment was verified using PCR. The rationale for this method is that the sequence beginning with the original stop codon should be different in chromosomes with and without inserted genes (Fig. 2). Three primers were included in the PCR reaction mix: two forward primers and one reverse primer. The primers were designed to give PCR products of different sizes. The size of the product from chromosomes containing the insert was designed to be ~0.5 % smaller than the size of the product from chromosomes lacking the insert. Yeast clones that are heterozygous for the insert should ordinarily produce PCR fragments of two different sizes, reflecting the fact that insertion would usually occur in only one of the two chromosomes.

→ = forward primer ← = reverse primer

F→
 AGGTCCTGGACTGACGATTACGCCTA
 ←R1

F→
 AGGTCCTGGAC--gene for GFP--gene for kanamycin resistance--CGATTACGCCTA
 ←R2

Figure 2. Example of verification of correct insertion of SFH-PCR fragment using PCR.

Materials and Methods

Strains, Media, and Culture Conditions

Saccharomyces cerevisiae strain W303, obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF), was used throughout this study. Yeast were maintained on 1.5% agar slants containing glucose-rich YPD (yeast extract/peptone/dextrose) medium: 1% yeast extract, 2% peptone, 2% dextrose. Reagents were from Difco. Before and after transfer into lipid-rich medium, cells were grown in liquid YPD (see below). Lipid-rich YPEO (yeast extract/peptone/ethanol/oleate) medium contained 1% yeast extract, 2% peptone, 2% ethanol, 0.2 % oleic acid (Sigma) and 0.02% Tween 80 (Sigma). Media used for transformed yeast expressing GFP fusion proteins (see below) also contained 50mg/L G418 antibiotic (Mediatech).

GFP-Transformation

The sequences of both *pxa1* and *pex3* were obtained from GenBank (NCBI). The plasmid (pFA6a-GFP(S65T)-kanMX6), containing the GFP-S65T variant of the gene for Green Fluorescent Protein and a gene for kanamycin resistance (*kan^r*), was generously

provided by Dr. Peter Philippsen, Biozentrum - University of Basel, Basel, Switzerland. The plasmid was isolated from the XL1-Blue strain of *E. coli* using a QIAprep kit (Qiagen). The *gfp* and *kan^r* genes bearing short flanking homology (SFH) sequences to the stop codons of the *pex3* and *pxa1* coding regions were prepared by PCR (Wach *et al.*, 1997, Yeast Resource Center), using the following primers: PXA-F (forward primer), AAAAGGTTGGGAAGATGAGAGGACGAAGCTACGGGAAAAGCTTGAAATTAT TGGTCGACGGATCCCCGGG; PXA-R (reverse primer), AATATATAAATTATATTCGCTAAATAAAATCTCTCCCTTTCTAGGGTGTTTAT CGATGAATTCGAGCTCG; PEX3-F, CGCCACGCTATACAGCAACTTTGGCGTCTCCAGCTCGTTTTTCCTC AAGCCTGGTCGACGGATCCCCGGG; PEX3-R, TACGCTATATATATATATATT CTGGTGTGAGTGTTCAGTACTTATTCAGAGAATCGATGAATTCGAGCTCG. The W303 strain of *S. cerevisiae* was transformed using a modification of the procedure of Gietz *et al.* (1995, Yeast Resource Center). Correct insertion of the GFP-S65T and *kan^r* genes was verified using PCR (Wach *et al.* 1998). The primers used for verification were INS-F, GATACTAACGCCGCC ATCCAG; INS-R, CCCGGGATCCGTCGACC; PXA1ver-F1, ACGCAATTAGTGTTGACATGGAAT; PXA1ver-R1, GTGTTGGCAAGACCAACAACACTGT; PEX3ver-F1, CCAAGAAAAACCTGGCCAAG; PEX3ver-R1, AAGTCACCATATCTGCGCTACGTT.

Transferring Medium

Cells were first transferred by loop from slants to liquid YPD and grown at 30°C on a shaker rotating at 200 rpm for 14 h in order to allow cells to reach the logarithmic

growth phase. After washing the cells twice with deionized water, they were inoculated at a density of 10^6 cells/ml into lipid-rich YPEO. The cells were grown in this medium at 30°C on the shaker for varying periods of time (see Results). In order to study the effect of transferring cells from lipid-rich to glucose-rich medium, cells were incubated in YPEO as described above for 20 h. The cells were then washed twice in deionized water and transferred into YPD. .

Microscopy and Photography

A Nikon Optiphot microscope, equipped with epifluorescence and differential interference contrast (DIC) optics. A CoolSNAP (Photomedia) digital camera was used for photography. All pictures were taken at using a Nikon PlanFluor 100X objective lens.

Results

Verification of Correct Insertion of SFH-PCR fragments

Integration of the SFH-PCR fragments containing genes for GFP and kanamycin resistance at the 3' ends of the *pex3* and *pxa1* coding regions was verified by PCR. If no insertion of the fragments into the genome had occurred, then a single band of slightly less than 500 bp (*pxa1*, 488 bp; *pex3*, 490 bp) would be present. If insertion into one of the diploid pair of chromosomes had occurred, then an additional fragment of 220-300 bp (*pxa1*, 229 bp; *pex3*, 289 bp) would be present. Both the *pxa1* and *pex3* transformant clones used in this study yielded PCR products of the predicted sizes, confirming correct insertion of the SFH-PCR fragments into the yeast genomes. Fig. 3 shows the results obtained for *pxa1*; *pex3* results are not shown. A third band (Fig. 3, lane 2), possibly formed by base pairing between different PCR products, was also present.

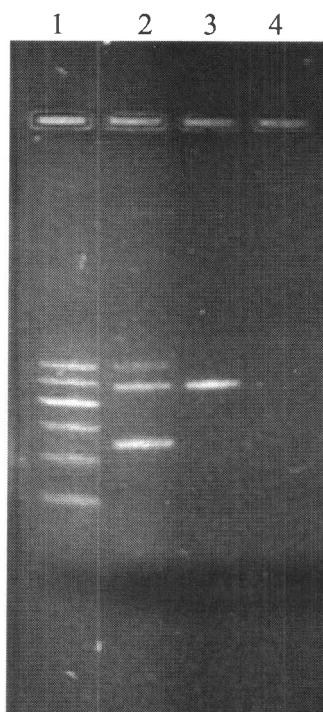


Figure 3. Verification of SFH-PCR fragment insertion into *pxa1*. Lane 1, DNA ladder, 100-600 bp. Lane 2, *pxa1* clone used in the study; Lane 3, no dash wild type (untransformed); Lane 4, - minus template DNA control

Incubation in YPEO medium.

Pex3p-GFP. Immediately after transfer from YPD to YPEO medium, fluorescence was faint and not concentrated in any particular region (Fig. 4a). The first noticeable difference was seen after 4 hours of incubation in the YPEO medium. At this time, fluorescence was still faint, but a band of slightly more intense fluorescence appeared to border the vacuole (Fig. 4b, arrow). After 6 hours in YPEO, the fluorescence was concentrated in more peripheral circular-oval structures (Fig. 4c). Finally after 48 hours, there were more numerous and larger fluorescent bodies (Fig. 4d).

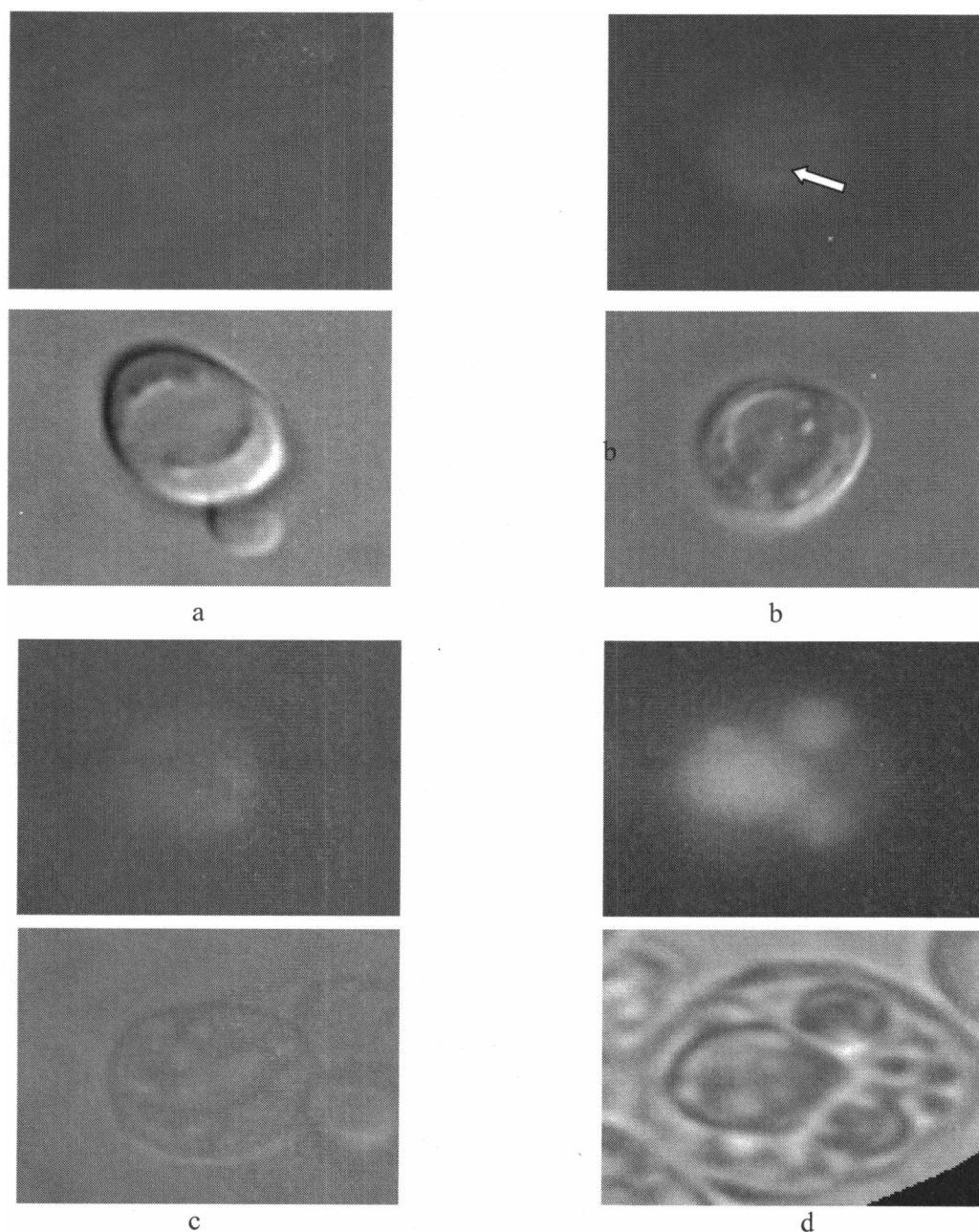
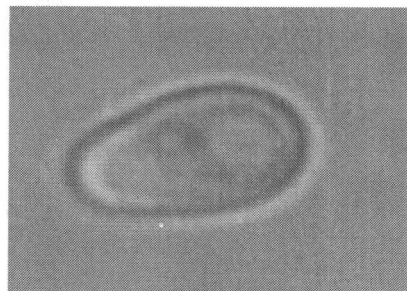
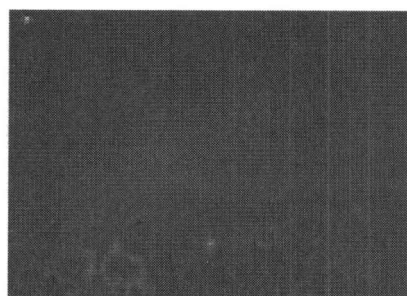


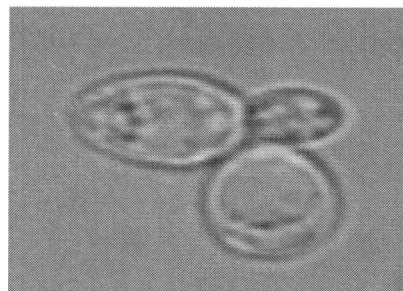
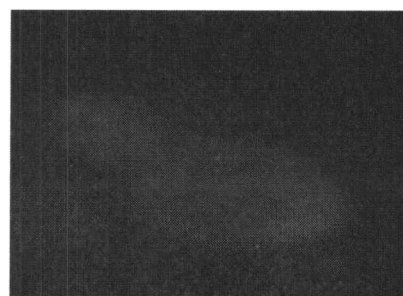
Figure 4. Pex3p-GFP fluorescence in YPEO medium. a, immediately after transfer from YPD to YPEO medium, the large circular structure in the center of the center in the lower photograph is the vacuole; b, 4 h in YPEO, arrow indicates slightly more intense fluorescence around vacuole; c, 6 h in YPEO; d, 48 h in YPEO. Top photograph, fluorescence; bottom photograph, DIC.

Pxa1p-GFP. Immediately after transfer into YPEO medium, there was little fluorescence visible in the cells (Fig. 5a). After 4 hours in YPEO, fluorescence appeared in patches in the peripheral cytoplasm. It was unclear whether the patches represented

discrete structures, whether the patches represented regions of cytoplasm between discrete structures, or whether they were some combination of the two (Fig. 5b). After 6 hours in YPEO, most of the fluorescence appeared concentrated in oval structures (Fig. 5c). After 48 hours, fluorescence within the circular-oval structures was more intense (Fig. 5d).



a



b

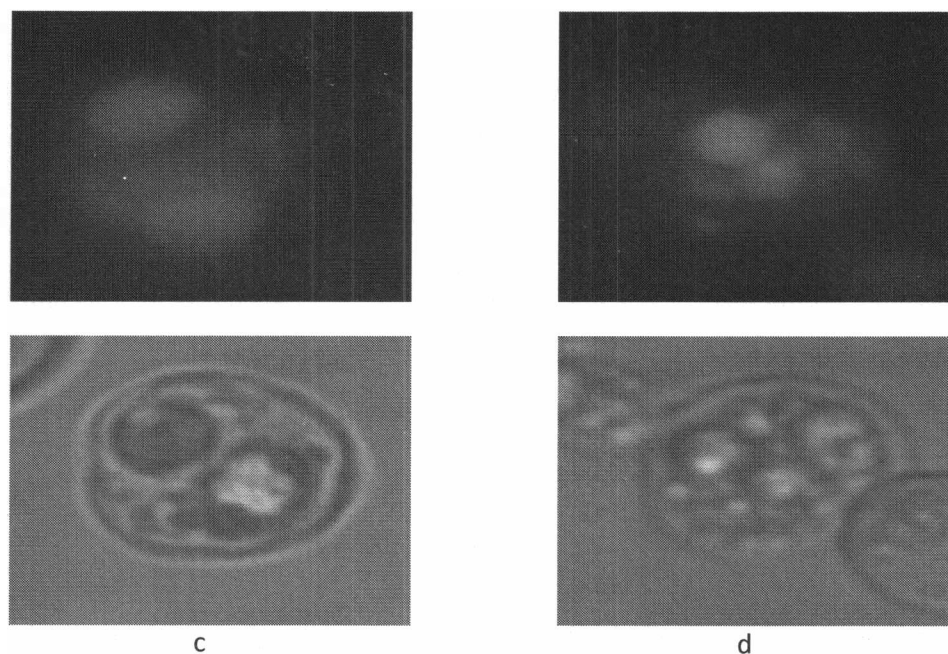


Figure 5. Pxa1p-GFP fluorescence in YPEO medium. a, immediately after transfer from YPD to YPEO medium; b, 4 h in YPEO; c, 6 h in YPEO; d, 48 h in YPEO. Top photograph, fluorescence; bottom photograph, DIC.

Incubation in YPD medium.

Pex3p-GFP. Yeast transformants that had been incubated in YPEO for 20 hours were transferred to YPD. Immediately after transfer, fluorescent bodies were conspicuous (Fig. 6a). After 30 min of incubation in YPD, the fluorescence had disappeared in most cells, but in some, it persisted as a ring in the peripheral cytoplasm (Fig.6b). After 4 hours of incubation, the fluorescence had disappeared (Fig.6c).

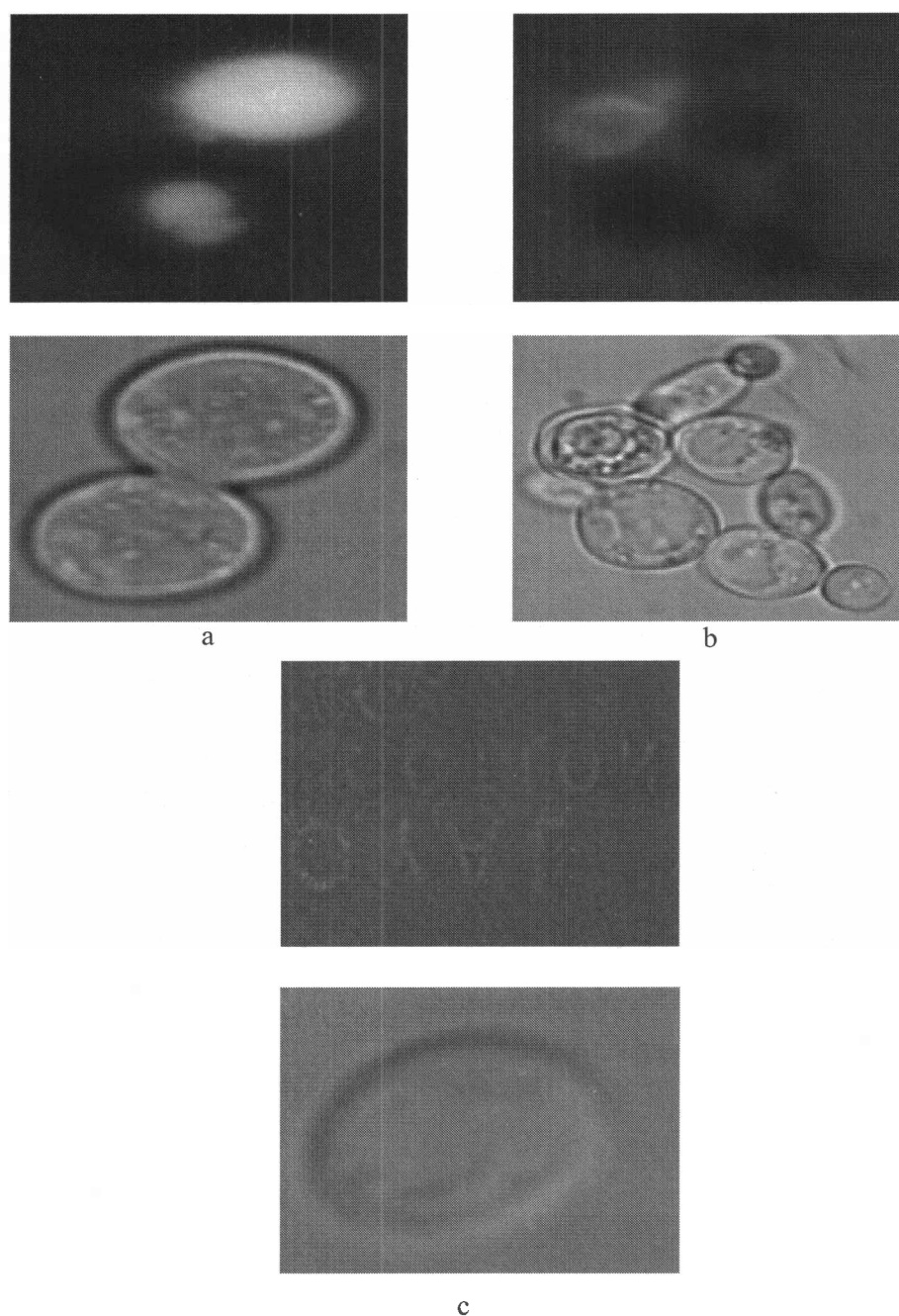


Figure 6. Pex3p-GFP fluorescence in YPD medium. a, immediately after transfer from YPEO to YPD medium; b, 30 min in YPD; c, 4 h in YPD. Top photograph, fluorescence; bottom photograph, DIC.

Pxalp-GFP. Immediately after the transfer from YPEO into YPD medium, fluorescence was concentrated in round-oval bodies of various sizes (Fig. 7a). After incubation in YPD for 30 min, a small amount of fluorescence remained in a centrally

located, oval region (Fig. 7b). After 90 min the fluorescence had disappeared (data not shown).

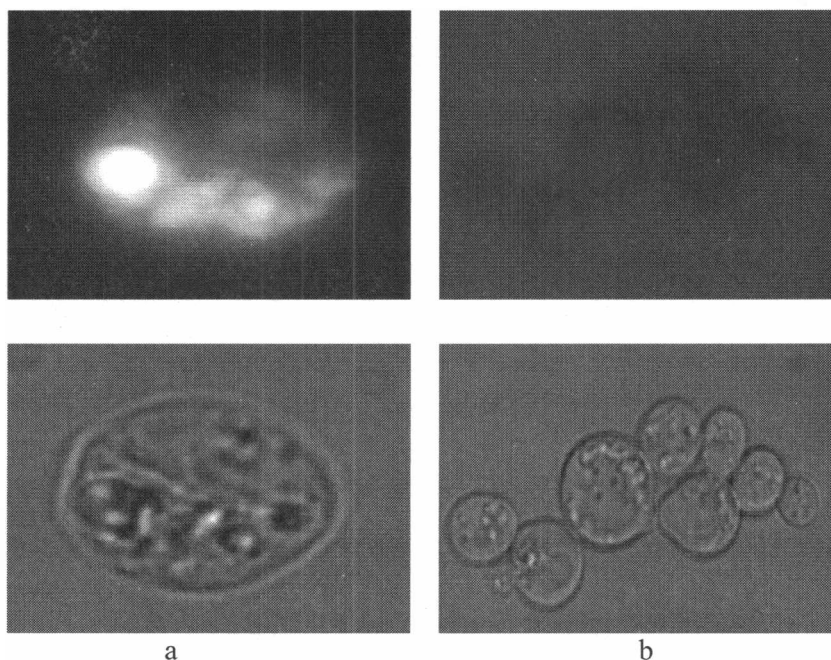


Figure 7. Pxa1p-GFP fluorescence in YPD. a, immediately after transfer from YPEO to YPD medium; b, 30 min in YPD; C, 4 h in YPD. Top photograph, fluorescence; bottom photograph, DIC.

Discussion

Peroxisomal Formation in YPEO medium

The objective of this research was to determine the effect of the culture medium composition on the localization of peroxisomal membrane proteins that had been labeled with GFP. Transfer of cells from YPD to YPEO medium resulted in a pronounced increase in fluorescence, which became concentrated in circular-oval bodies of various sizes over a period of 4-6 h. That the fluorescent bodies were peroxisomes is suggested by the observations that the gene for GFP was inserted correctly into the *pex3* and *pxa1* genes (Fig. 1) and that GFP is the only molecule in the cells that fluoresces. Cells

incubated in YPEO medium accumulate lipid droplets in addition to peroxisomes (Thieringer *et al.* 1991), but oleic acid droplets do not fluoresce (data not shown), arguing against lipid droplets as the source of the fluorescence. The observation that the sizes and shapes of the fluorescent bodies produced with Pex3p-GFP and Pxa1p-GFP are similar furthermore suggests that the two GFP-labeled proteins ended up in the same compartment. While it is conceivable that both GFP-labeled proteins accumulated in compartments other than peroxisomes, this possibility seems less likely than the possibility that both proteins were concentrated in peroxisomes.

Several groups have demonstrated formation of new peroxisomes in the yeast, *S. cerevisiae*, when yeast cells are transferred to a medium that forces the cells to use fatty acids as the energy source (Palmieri *et al.*, 2001; Thieringer *et al.*, 2001; Nair *et al.*, 2004); but in these reports, the majority of newly formed peroxisomes appeared smaller than the large structures that formed in my experiments. The reason for the larger size of some of the peroxisomes in my study is not clear.

The concentration of Pex3p-GFP fluorescence in an arc around the vacuole 30 min after transfer into YPEO is consistent with at least some Pex3p being incorporated first into the ER during *de novo* peroxisome formation (Hoepfner *et al.*, 2005). The diffuse fluorescence surrounding the more concentrated band could be due either to membrane bound Pex3p-GFP located above or below the focal plane or to cytosolic protein. Pxa1p-GFP fluorescence, 30 min after transfer into YPEO medium, was diffuse, an observation consistent with the presence of Pxa1p in the cytosol; ; however, I cannot rule out the possibility that at least some of the Pxa1p-GFP fluorescence is associated with a membrane-bounded compartment.

Degradation of Peroxisomes in YPD medium following incubation in YPEO medium

Fluorescence due to both Pex3p-GFP and Pxa1p-GFP disappeared rapidly after transfer back to YPD. There are two mechanisms through which peroxisomal degradation, or pexophagy, can occur: macropexophagy, which is when the peroxisomes are individually sequestered and the resulting phagosome then fuses with the vacuole, and micropexophagy, which is when clusters of unsequestered peroxisomes are directly internalized into the vacuole (Monastyrska and Klionsky, 2006). In this study the mechanism of degradation could not be determined; however, the ring of fluorescence observed with Pex3p-GFP and the faint, centrally located zones of fluorescence observed with Pxa1p-GFP are consistent with involvement of the vacuole which is seen in both macropexophagy and micropexophagy.

Conclusions

I succeeded in producing GFP transformants for two peroxisomal genes. I also confirmed that switching to lipid-rich medium induced peroxisome formation and demonstrated that switching the peroxisomes back into the glucose medium triggered a rapid degradation. Evidence consistent with the hypotheses that Pex3p moves from the ER to the peroxisomal membrane and that Pxa1p is transported from the cytosol to the membrane was obtained, but the overall support was weak. This system holds promise for future studies.

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