PpATG9 Encodes a Novel Membrane Protein That Traffics to Vacuolar Membranes, Which Sequester Peroxisomes during Pexophagy in *Pichia pastoris*

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When *Pichia pastoris* adapts from methanol to glucose growth, peroxisomes are rapidly sequestered and degraded within the vacuole by micropexophagy. During micropexophagy, sequestering membranes arise from the vacuole to engulf the peroxisomes. Fusion of the sequestering membranes and incorporation of the peroxisomes into the vacuole is mediated by the micropexophagy-specific membrane apparatus (MIPA). In this study, we show the *P. pastoris* ortholog of Atg9, a novel membrane protein is essential for the formation of the sequestering membranes and assembly of MIPA. During methanol growth, GFP-PpAtg9 localizes to multiple structures situated near the plasma membrane referred as the peripheral compartment (Atg9-PC). On glucose-induced micropexophagy, PpAtg9 traffics from the Atg9-PC to unique perivacuolar structures (PVS) that contain PpAtg11, but lack PpAtg2 and PpAtg8. Afterward, PpAtg9 distributes to the vacuole surface and sequestering membranes. Movement of the PpAtg9 from the Atg9-PC to the PVS requires PpAtg11 and PpVps15. PpAtg2 and PpAtg7 are essential for PpAtg9 trafficking from the PVS to the vacuole and sequestering membranes, whereas trafficking of PpAtg9 proceeds independent of PpAtg1, PpAtg18, and PpVac8. In summary, our data suggest that PpAtg9 transits from the Atg9-PC to the PVS and then to the sequestering membranes that engulf the peroxisomes for degradation.

INTRODUCTION

Cellular activities are regulated and maintained as a well-controlled balance between protein synthesis and degradation of essential proteins and enzymes. Protein lifetimes can last from minutes to days, with a majority of the cellular proteins being degraded in a lytic compartment such as the lysosome or vacuole. Autophagy is the primary pathway for the delivery of endogenous proteins and organelles to this compartment (Levine and Klionsky, 2004; Klionsky, 2005). There exist a number of autophagic pathways in yeast: micro- and macro-autophagy, micro- and macro-pexophagy, cytoplasm-to-vacuole targeting pathway (CVT), and vacuole import and degradation pathway. The CVT pathway is constitutive, whereas the other pathways are regulated by environmental signals. For example, autophagy is enhanced when cells are deprived of nutrients such as amino acids and

glucose, whereas pexophagy is activated when methylotrophic yeasts adapt from growth on methanol medium to growth on glucose or ethanol medium. Autophagy sequesters cytosolic proteins and organelles nonselectively, whereas pexophagy is responsible for the selective degradation of peroxisomes. Despite their differences, these pathways share a number of common molecular events. For example, Atg7/Gsa7/Apg7, Atg1/Gsa10/Apg1/Aut3, Atg2/Gsa11/Apg2, Atg18/Gsa12/Cvt18, and Vps15 are required for pexophagy, autophagy, and CVT pathways, whereas Atg11/Gsa9/Cvt9 and Vac8 are essential for pexophagy and CVT pathways, but not autophagy (Stasyk *et al.*, 1999; Yuan *et al.*, 1999; Scott *et al.*, 2000; Guan *et al.*, 2001; Kim *et al.*, 2001; Strømhaug *et al.*, 2001; Strømhaug and Klionsky, 2001; Wang *et al.*, 2001; Huang and Klionsky, 2002; Abeliovich *et al.*, 2003). Because many autophagy genes and their orthologues have been identified using different yeast models, the nomenclature for these genes had become confusing. Therefore, we will utilize the ATG nomenclature for those genes uniquely essential for autophagy (We will conform to the ATG nomenclature; all the autophagy-related [ATG] genes will contain a genus and species prefix, e.g., PpAtg for the *P. pastoris* and ScAtg for the *S. cerevisiae* homologues; Klionsky *et al.*, 2003).

Pichia pastoris is able to synthesize enzymes to assimilate methanol for energy and growth. These enzymes such as alcohol oxidase (AOX) are housed primarily in the peroxisomes (Tuttle *et al.*, 1993). When these cells adapt from methanol to ethanol or glucose, the now superfluous peroxisomes are rapidly degraded by autophagic events. During ethanol adaptation, peroxisomes are individually and

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Abbreviations used: AOX, alcohol oxidase; FM 4-64, N-(triethylammoniumpropyl)-4-(p-diethylaminophenyl)hexatrienyl pyridinium dibromide; TCA, trichloroacetic acid; GFP, green fluorescent protein; BFP, blue fluorescent protein; mRFP, monomeric red fluorescent protein; GSA, glucose-induced selective autophagy; REMI, restriction enzyme-mediated integration; CVT, cytoplasm-to-vacuole targeting; Atg9-PC, Atg9 peripheral compartment; PVS, perivacuolar structure; MIPA, micropexophagy-specific membrane apparatus; PAS, pre-autophagosome structure.

selectively sequestered into autophagosomes that then fuse with the vacuole. This process is called macropexophagy. A second venue for selective peroxisome degradation occurs during glucose adaptation. Micropexophagy proceeds by a mechanism whereby the vacuolar membrane invaginates, resulting in protrusions that surround and engulf the entire cluster of peroxisomes (Tuttle *et al.*, 1993; Sakai *et al.*, 1998). Both pathways result in the degradation of peroxisomes within the vacuole by proteolytic enzymes.

Pichia pastoris is a particularly good genetic model for the study of pexophagy because of its ability to rapidly and selectively degrade peroxisomes (i.e., AOX) when adapting from methanol to glucose medium. The sequestration and degradation of peroxisomes during glucose adaptation is completed within 6 h (Tuttle and Dunn, 1995). Based on the movements of the vacuole that can be easily observed by fluorescence and electron microscopy, glucose-induced micropexophagy has been described as a multistage process that includes: signaling events (vacuole round), early sequestration events (vacuole indented), intermediate sequestration events (vacuole indented with short armlike processes), late sequestration events (vacuole indented with long armlike processes), and vacuole degradation (vacuole with autophagic bodies; Sakai *et al.*, 1998; Strømhaug *et al.*, 2001; Mukaiyama *et al.*, 2002). We and others have utilized genetic screens to identify a number of unique proteins required for peroxisome degradation in *P. pastoris* (Tuttle and Dunn, 1995; Sakai *et al.*, 1998; Strømhaug *et al.*, 2001). To better understand the functions of these proteins, we examined the vacuole morphology upon glucose-induced pexophagy. For example, PpVps15 (Paz13), PpAtg11 (Gsa9), and PpAtg18 (Gsa12) appear to act at early sequestration events (Stasyk *et al.*, 1999; Guan *et al.*, 2001; Mukaiyama *et al.*, 2002), PpAtg2 (Gsa11) and PpAtg7 (Gsa7/Paz12) at intermediate sequestration events (Yuan *et al.*, 1999; Strømhaug *et al.*, 2001), and PpAtg1 (Paz1/Gsa10) at late sequestration events (Mukaiyama *et al.*, 2002). Many of these proteins are structurally and functionally homologous to those proteins essential for autophagy and CVT pathways in *Saccharomyces cerevisiae* (Habibzadegah-Tari and Dunn, 2003; Klionsky *et al.*, 2003). Furthermore, structural homologues of these proteins can be found in various invertebrates and vertebrates including humans (Klionsky *et al.*, 2003).

In this study, we have sequenced and characterized a unique membrane protein that is required for both pexophagy and autophagy in *P. pastoris*. Based on vacuole morphology during glucose adaptation, we project that PpAtg9 is required for an early sequestration event. In growing cells, this protein localizes to unique foci near the cell periphery. During glucose-induced pexophagy, PpAtg9 traffics from these vesicles to perivacuolar structures that appear to adjoin the vacuole and then to the sequestering membranes that arise from the vacuole. The trafficking of PpAtg9 requires PpAtg7, PpAtg11, PpAtg2, and PpVps15, but not PpAtg1, PpAtg18, or PpVac8. Furthermore, our data suggest that the transfer of PpAtg9 to the vacuole is a prerequisite for the formation of those sequestering membranes that engulf the peroxisome for incorporation into the vacuole for degradation.

MATERIALS AND METHODS

Yeast Strains and Media

The yeast strains used in this study are listed in Table 1 and were routinely cultured at 30°C in YPD (1% Bacto yeast extract, 2% Bacto peptone, and 2% dextrose). *P. pastoris* was grown in YNM (0.67% yeast nitrogen base, 0.4 mg/l biotin, and 0.5% methanol) to induce peroxisome biogenesis. The degradation

of peroxisomes was induced when cells grown in YNM were transferred to YND (0.67% yeast nitrogen base, 0.4 mg/l biotin, and 2% glucose) or YNE (0.67% yeast nitrogen base, 0.4 mg/l biotin, and 0.5% ethanol). Nitrogen starvation medium contained 0.17% yeast nitrogen base (without amino acids and NH_4SO_4) and 2% glucose. All media contained 2% agar when made as plates. Histidine or arginine or both were added at 40 $\mu\text{g}/\text{ml}$ when needed. Vector amplification was done in *Escherichia coli* (DH5 α) cultured at 37°C in LB (0.5% Bacto yeast extract, 1% Bacto tryptone, and 1% NaCl) with ampicillin (100 $\mu\text{g}/\text{ml}$). Zeocin was added at 25 $\mu\text{g}/\text{ml}$ when culturing DH5 α and 100 $\mu\text{g}/\text{ml}$ when culturing *P. pastoris*.

Yeast Transformation

Cells grown overnight in YPD to an optical density (OD_{600}) of 1.0 were harvested and treated with 10 mM dithiothreitol (DTT) in YPD containing 25 mM HEPES, pH 8, for 15 min at 30°C. The cells were washed twice in ice-cold water and once in 1 M sorbitol and then resuspended into 1 M sorbitol. Cells (40 μl) were mixed with 0.2–1 μg of linearized vector and transferred to a 0.2-cm gap cuvette (Bio-Rad Laboratories, Hercules, CA), and the DNA was introduced by electroporation at 1.5 kV, 25 μF , 400 Ω (Gene Pulser, Bio-Rad Laboratories). The cells transformed with vectors containing the His4 gene were transferred to plates containing 0.67% yeast nitrogen base without amino acids, 2% glucose, 1 M sorbitol, 0.4 mg/L biotin, and 2% agar and incubated at 30°C for 3–5 d before colonies appeared. Cells transformed with vectors containing the zeocin resistance gene (zeo^R) were grown on YPD with 100 $\mu\text{g}/\text{ml}$ zeocin.

Isolation of *gsa* Mutants and Cloning of GSA Genes by Restriction Enzyme-mediated Integration Mutagenesis

Mutagenesis was performed by randomly inserting the pREMI-Z vector (restriction enzyme-mediated integration [REMI]; provided by Dr. Ben Glick, University of Chicago) that contained the zeocin resistance gene into the genome of *P. pastoris* as previously described (Strømhaug *et al.*, 2001). Those *gsa* mutants caused by disruption of gene expression were identified by direct colony assays (Strømhaug *et al.*, 2001). The site of insertion of the pREMI-Z and identification of the disrupted gene was done as described (Strømhaug *et al.*, 2001). Based on the genomic sequences around the pREMI-Z insertion site that was isolated along with the pREMI-Z vector from the R19 mutant, GSA14 was cloned and sequenced from genomic DNA using a linker-mediated PCR method previously described (van Der Wel *et al.*, 2001). We were able to completely assemble the GSA14 gene (National Center for Biotechnology Information [NCBI] accession number AY075105) and show it encodes a protein homologous to ScAtg9 of *S. cerevisiae*, using the adapted ATG nomenclature for those genes uniquely essential for autophagy (Klionsky *et al.*, 2003). A search of the NCBI database revealed structural homologues of PpAtg9 and ScAtg9 in *Schizosaccharomyces pombe* (NP_596247), *Arabidopsis thaliana* (NP_180684), *Neurospora crassa* (XP_331198), *Drosophila melanogaster* (NP_611114), *Caenorhabditis elegans* (NP_503178), and *Homo sapiens* (NP_076990; Yamada *et al.*, 2005). The alignment of these proteins reveals a large central region (190–679 residues) of homology containing at least five putative transmembrane domains. The pREMI-Z disruption of PpAtg9 occurred after the first putative transmembrane domain at aspartic acid 245. Using this approach, we have isolated R2, R12, R13, and R22 mutants. The R2 (*his4 Ppatg18-1::zeo^R*) and R13 (*his4 Ppatg11-1::zeo^R*) mutants have been described elsewhere (Guan *et al.*, 2001; Kim *et al.*, 2001; Strømhaug *et al.*, 2001). The R12 mutants had the pREMI-Z inserted into the PpATG1 gene loci. The R22 (*his4 Ppatg2-2::zeo^R*) and WDK011 (*his4 Ppatg2Δ::zeo^R*) mutants have been characterized previously (Strømhaug *et al.*, 2001). The *Ppatg7/gsa7* mutants have been previously described (Yuan *et al.*, 1999). The null mutant of PpVps15 was provided by Dr. J. Clegg (Keck Graduate Institute; Stasyk *et al.*, 1999). PpVAC8 was cloned from genomic DNA using degenerate primers combined with linker-mediated PCR (van Der Wel *et al.*, 2001). The entire gene was sequenced and assembled (NCBI accession number AY886543). A *Ppvac8* null mutant was constructed by replacing the entire gene (–7 base pairs through 1669 base pairs) with the zeo^R gene driven by the TEF1 promoter. The null mutants were selected on YPD plates containing 100 $\mu\text{g}/\text{ml}$ zeocin and replica-plated to YNM plates. Null mutants were identified by direct colony assay (see below) and verified by PCR using primers flanking PpVAC8 and within the zeo^R gene (unpublished data).

Measurements of Alcohol Oxidase (AOX) and Endogenous Protein Degradation

The direct colony and liquid medium assays to detect and measure the degradation of peroxisomal AOX was performed as previously described (Strømhaug *et al.*, 2001). Briefly, cells were grown in YNM for 40 h. At which time, glucose (2%) or ethanol (0.5%) was added. Aliquots of cells (2 ml of $\text{OD}_{600} = 1.4$) at 0 and 6 h of glucose or ethanol adaptation were pelleted and resuspended in 1 ml 20 mM Tris, pH 7.5, containing 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 $\mu\text{g}/\text{ml}$ pepstatin A, and 0.5 $\mu\text{g}/\text{ml}$ leupeptin. The cells were then lysed by vortexing in the presence of glass beads (425–600 μm). The glass beads and cellular debris were removed by centrifugation and AOX measured (Tuttle and Dunn, 1995;

Table 1. *Pichia pastoris* strains

Name	Genotype	Reference
GS115	<i>his4</i>	(Cregg <i>et al.</i> , 1985)
PPF1	<i>arg4 his4</i>	(Yuan <i>et al.</i> , 1997)
SMD1163	<i>his4 pep4 prb1</i>	(Tuttle and Dunn, 1995)
DMM1	GS115::pDM1 (P _{AOX1} BFP-SKL ₊ , <i>zeo</i> ^R)	(Kim <i>et al.</i> , 2001)
WDY7	<i>his4 Ppatg7</i>	(Yuan <i>et al.</i> , 1999)
WDKO7	PPF1 <i>Ppatg7Δ::ARG4</i>	(Yuan <i>et al.</i> , 1999)
WDKO11	GS115 <i>Ppatg2Δ::zeo</i> ^R	(Strømhaug <i>et al.</i> , 2001)
R2	GS115 <i>Ppatg18-1::zeo</i> ^R	(Strømhaug <i>et al.</i> , 2001)
R12	GS115 <i>Ppatg1-1::zeo</i> ^R	(Strømhaug <i>et al.</i> , 2001)
R13	GS115 <i>Ppatg11-1::zeo</i> ^R	(Kim <i>et al.</i> , 2001)
R19	GS115 <i>Ppatg9-1::zeo</i> ^R	(Strømhaug <i>et al.</i> , 2001)
R22	GS115 <i>atg2-1::zeo</i> ^R	(Strømhaug <i>et al.</i> , 2001)
Ppvps15Δ	PPF1 <i>his4 Ppvps15Δ::ARG4</i>	(Stasyk <i>et al.</i> , 1999)
S7-GFPx3	PPF12 <i>arg4 his4::pPOP-S7-GFPx3</i> (P _{Sec7} PpSec7-GFPx3)	(Bevis <i>et al.</i> , 2002)
Sec13-GFP	PPF12 <i>arg4 his4::pPOP-SEC13-GFP</i> (P _{Sec13} PpSec13-GFP)	(Bevis <i>et al.</i> , 2002)
WDY53	<i>arg4 his4 vac8Δ::zeo</i> ^R	This study
WDY70	DMM1 <i>his4::pWD21</i> (P _{GAPDH} GFP-PpAtg8, HIS4)	This study
WDY71	R19 <i>his4::pWD21</i> (P _{GAPDH} GFP-PpAtg8, HIS4)	This study
WDY75	AJM26 <i>his4::pWD21</i> (P _{GAPDH} GFP-PpAtg8, HIS4)	This study
WDY78	R19 <i>his4::pAJM9</i> (P _{GAPDH} mRFP-PpAtg9, HIS4)	This study
TC1	GS115 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC3	DMM1 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC4	WDY7 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC5	WDKO7 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC6	R13 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC7	R12 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC8	R22 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC9	R2 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC10	R19 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC11	PpVsp15Δ <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC12	WDY53 <i>arg4 his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
TC14	R19 <i>his4::pTC2</i> (P _{GSA14} PpATG9-GFP, HIS4)	This study
TC16	R19 <i>his4::pTC3</i> (P _{GAPDH} PpATG9-GFP, HIS4)	This study
TC19	WDKO11 <i>his4::pTC1</i> (P _{GAPDH} GFP-PpATG9, HIS4)	This study
ASK2	GS115 <i>his4::pHA-G14</i> (P _{GSA14} HA-PpATG9, HIS4)	This study
ASK3	R19 <i>his4::pHA-G14</i> (P _{GSA14} HA-PpATG9, HIS4)	This study
ASK4	R19 <i>his4::pGFP-G14(ΔL3)</i> (P _{GAPDH} GFP-PpAtg9ΔL3, HIS4)	This study
ASK5	R19 <i>his4::pGFP-G14(ΔN)</i> (P _{GAPDH} GFP-PpAtg9ΔN, HIS4)	This study
AJM18	S7-GFPx3 <i>arg4 his4::pAJM6</i> (P _{GAPDH} mRFP-PpATG9, <i>zeo</i> ^R)	This study
AJM19	Sec13-GFP <i>arg4 his4::pAJM6</i> (P _{GAPDH} mRFP-PpATG9, <i>zeo</i> ^R)	This study
AJM22	GS115 <i>his4::pPS69</i> (P _{GAPDH} GFP/HA-PpATG2, HIS4)	This study
AJM24	GS115 <i>his4::pWD17</i> (P _{GAPDH} GFP-PpATG9, <i>zeo</i> ^R)	This study
AJM25	AJM22::pAJM6 (P _{GAPDH} mRFP-PpATG9, <i>zeo</i> ^R)	This study
AJM26	GS115 <i>his4::pAJM6</i> (P _{GAPDH} mRFP-PpATG9, <i>zeo</i> ^R)	This study
AJM27	AJM24 <i>his4::pIB2-dsRED-HDEL</i> (P _{GAPDH} dsRFP-HDEL, HIS4)	This study
AJM32	AJM26 <i>his4::pPS64</i> (P _{GAPDH} GFP/HA-PpATG11, HIS4)	This study
AJM33	AJM26 <i>his4::pPS55-G12</i> (P _{GAPDH} GFP/HA-PpAtg18, HIS4)	This study
AJM44	AJM26 <i>his4::pAJM3</i> (P _{ADH} CoxIV-GFP, HIS4)	This study

Yuan *et al.*, 1999). The degradation of endogenous proteins during nitrogen starvation was performed as described previously (Strømhaug *et al.*, 2001). Briefly, cellular proteins were radiolabeled with ¹⁴C-valine for 16 h, and the cells switched to nitrogen starvation medium containing 0.17% yeast nitrogen base (without amino acids and NH₄SO₄) and 2% glucose and supplemented with 10 mM valine. The rates of protein degradation were calculated from the slopes of the linear plots of TCA-soluble radioactivity over 2–24 h of chase.

Western Blot Analysis

Cells (2 ml) from cultures grown to an optical density (OD₆₀₀) of 1.4 were collected by centrifugation and prepared for SDS-PAGE as previously described (Tuttle and Dunn, 1995). The cells were lysed in 67 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 1.5% DTT solution, and 2 μl of a proteinase inhibitor cocktail (200 mM PMSF, 14.5 mM pepstatin A, 10.5 mM leupeptin in dimethyl sulfoxide) by vortexing with glass beads. The proteins were separated by SDS-PAGE and then transferred to nitrocellulose by Trans-Blot SemiDry Transfer Cell (Bio-Rad Laboratories) for 1 h. The blots were blocked in 5% nonfat dried milk in phosphate-buffered saline and then incubated with mouse anti-AOX or rabbit anti-HA antibody (Covance, Princeton, NJ). After incubation with secondary goat anti-mouse antibody

conjugated with HRP (Covance), the blots were washed and HRP was detected using ECL-plus (Amersham, Piscataway, NJ) and quantified using the Typhoon 9400 laser scanner (Molecular Dynamics, Sunnyvale, CA).

Construction of PpAtg Expression Vectors

The gene for the green fluorescent protein (GFP) was inserted behind the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter into the *Eco*RI site of pIB2 (Sears *et al.*, 1998). The resulting expression vector, pPS55, was then used to construct the GFP fusion protein of PpAtg9 being expressed by the constitutive and glucose-inducible GAPDH promoter. *PpATG9* was amplified from genomic DNA by PCR with EnzyPlus polymerase (Enzygol, Boulder, CO) using a forward primer of 5'-CTCTCACATTGTCCGTACCATGCATAAGAATAACACGAC-3' that contained a *Kpn*I site. The reverse primer 5'-GTTTGGACTCGAGGGTACTAATGCTTCATT-3' contained an *Xho*I site. *PpATG9* was inserted behind the *GFP* gene in pPS55. A second expression vector called pTC2 was created by inserting *PpATG9* with its endogenous promoter in front of *GFP* in pWD3 whereby *GFP* had been inserted into the *Sph*I site of pIB1 (Sears *et al.*, 1998). *PpATG9* was amplified from genomic DNA by PCR using a forward primer 5'-GCAGGCTAGGGTACCGGTACTGGCACATT-3' that contained *Kpn*I site and a reverse primer

5'-CAAGATCTATGCTCGAGAACAATAATGCCTTATGCTGTGACTAA-3' with an *XhoI* site. This product was then inserted into the *KpnI* and *XhoI* sites of pWD3, the resulting fusion construct verified by sequencing, and the vector used to transform R19 (*his4 atg9::zeo^R*) cells. pTC3 was made using *PpATG9* with a GAPDH promoter in place of the *PpATG9* endogenous promoter by amplifying it from genomic DNA using a forward primer 5'-CTCTCACATTGTCGGTACCATGCATAAGAATAACACGAC-3' containing a *KpnI* site and a reverse primer 5'-CAAGATCTATGCTCGAGAA-CAAATAATGCCTTATGCTGTGACTAA-3' with an *XhoI* site. It was inserted in front of the *GFP* gene of pWD4, which had been inserted into the *SphI* site of pIB2 (Sears *et al.*, 1998). Two additional expression vectors were made by inserting the *PpATG9* gene behind the *GFP* and *mRFP* genes that were inserted into the *EcoRI* site of pGAPZ (Invitrogen, San Diego, CA). The gene for *mRFP* was kindly provided by Dr. R. Y. Tsein (University of California at San Diego; Campbell *et al.*, 2002). *PpATG9* was amplified from genomic DNA using a forward primer 5'-CTCTCACATTGTCGGTACCATGCATAAGAATAACACGAC-3' containing a *KpnI* site and a reverse primer 5'-CATTATTATTCAGACCGCGGAATGAAAA-3' with a *SacII* site. The PCR product was then cut with *KpnI* and *SacII* and inserted into pGAPZ-GFP and pGAPZ-RFP resulting in pWD17 and pAJM6 vectors, respectively. GFP-*PpAtg9*(Δ N) lacking the N-terminus upstream of the first transmembrane region and GFP-*PpAtg9*(Δ L3) lacking a highly conserved third loop between the 3rd and 4th transmembrane regions was constructed by PCR. A *PpAtg9* gene product lacking M1 through Y211 was amplified from genomic DNA by PCR using a forward primer 5'-CGACTATGTTACCGGAAATGGAT-TCAA-3' with a *KpnI* site and a reverse primer 5'-GTTTGGACTCGAGGG-TACTAATGCTTCATT-3' with an *XhoI* site. The resulting gene was inserted into the *KpnI* and *XhoI* sites of pPS55. A second *PpAtg9* gene product lacking T471 through A523 was constructed by independently amplifying the gene fragments upstream and downstream of the deletion. The upstream region of the *PpATG9* gene was amplified using a forward primer of 5'-CTCTCACAT-TGTCGGTACCATGCATAAGAATAACACGAC-3' that contained a *KpnI* site and a reverse primer of 5'-CCGAGGATTAAGCTTAAAAATCGTA-AAAAA-3' containing a unique *HindIII* site. The downstream region of the *PpATG9* gene was amplified using a forward primer of 5'-CTACAAC-GAAGCTTCTGAAGTTCATCATGT-3' containing an *HindIII* site and a reverse primer 5'-GTTTGGACTCGAGGGTACTAATGCTTCATT-3' containing an *XhoI* site. These two fragments were digested with the appropriate restriction enzymes and ligated into the *KpnI* and *XhoI* sites of pPS55. The expression vector, pWD21, contained *PpAtg8* with a GFP at its N-terminus. *PpAtg8* was amplified from genomic DNA by PCR using a forward primer of 5'-CCATGAATCCATGCGATCGCAATTTAAAGACGAACA-3' containing an *EcoRI* site and a reverse primer of 5'-GGCACTACTCGAGTTATTAT-TCAATCTCTCAACACCTGGAA-3' containing an *XhoI* site. *PpAtg8* was inserted behind the *GFP* gene in pPS55. The final expression vector, pASK1, contained *PpAtg9* with an HA tag at its N-terminus driven by the endogenous promoter of *PpATG9*. This construct was made by PCR of the *PpATG9* promoter and open reading frame separately, which was then followed by a PCR amplification of the entire gene including promoter. The promoter was amplified using a forward primer of 5'-GCAGGCTAGGGTACCGGTACTG-GCACATT-3' containing a *KpnI* site and a reverse primer of 5'-GCGTAAT-CTGGAACATCGTATGGATACATTCAATCGACAATGTGAGAGATTTCAG-TGAAG-3' containing the HA tag. The open reading frame was amplified using a forward primer of 5'-TGATCCATACGATGTTCAGATTACCG-GATGCATAAGAATAACACGACATTTTATCC-3' containing an HA tag (MYPYDVPDYA) insertion in front of the *PpAtg9* start codon and a reverse primer of 5'-GTCAGACTCCAACTCGAGTTCATTTTCAA-3' containing an *XhoI* site. Finally, the entire gene with promoter was amplified from the above products by PCR using forward 5'-GCAGGCTAGGGTACCGGTACTG-GCACATT-3' and reverse 5'-GTCAGACTCCAACTCGAGTTCATTTTCAA-3' primers. The resulting product was cut with *KpnI* and *XhoI* and inserted into pIB1. The CoxIV signal sequence plus the upstream ADH1 promoter was excised from pCC4 by cutting with *EcoRV* and *XbaI* (Campbell and Thorsness, 1998). The fragment was then inserted into the *SmaI* and *SpeI* sites upstream of the *GFP* gene of pWD3, which had been constructed by inserting the *GFP* gene into the *SphI* site of pIB3 (Sears *et al.*, 1998). The resulting expression vector, pAJM3, produced a GFP protein that was targeted to the mitochondria. The construction of pPS55-G12, pPS64, and pPS69 vectors have been previously described (Guan *et al.*, 2001; Kim *et al.*, 2001; Strømhaug *et al.*, 2001) and pPOP-S7-GFPX3, pPOP-SEC13-GFP, and pIB2-DsRED-HDEL were provided by Dr. Ben Glick (University of Chicago; Bevis *et al.*, 2002). For transformation, these vectors were first linearized by cutting either within the *HIS4* gene with *StuI* or *Sall* or within the *PpATG9* gene with *SacI*.

Fluorescence microscopy and FM 4-64 Labeling

Cells expressing GFP or RFP fusion proteins were grown in either YPD for 24 h or YNM for 20 h. Cells grown on YNM medium were then transferred to YND or YNE for 1–4 h. FM 4-64 (Molecular Probes, Eugene, OR) was added to a final concentration of 20 μ g/ml and the cells incubated for 2–12 h. The cells were washed of unbound FM 4-64 and examined immediately using a Zeiss Axiophot fluorescence microscope (Thornwood, NY). Image capture was done using SPOT camera (Diagnostics Instruments, Sterling Heights, MI) interfaced with IP Lab software.

Electron Microscopy

The cellular ultrastructure of *PpAtg9* mutants was examined as previously described (Tuttle and Dunn, 1995). Briefly, cells grown in YNM or grown in YNM and adapted to YND or YNE were harvested by centrifugation, washed in water, and fixed in 1.5% KMnO₄ in veronal-acetate buffer (28 mM sodium acetate, 28 mM sodium barbital, pH 7.6) for 20 min at 22°C (Veenhuis *et al.*, 1983). The specimens were dehydrated with increasing concentrations of ethanol and infiltrated with POLY/BED 812 (Polysciences, Warrington, PA) with accelerator 2,4,6-tri(dimethylaminomethyl) phenol (DMP-30, Polysciences) for 2 d at 22°C under vacuum. After polymerization of the resin, the samples were sectioned and examined using a JEOL 100CX transmission electron microscope (Peabody, MA).

RESULTS

PpAtg9 Mutants Are Defective in Glucose-induced Pexophagy and Starvation-induced Autophagy

P. pastoris can assimilate methanol for growth by synthesizing peroxisomal enzymes such as alcohol oxidase (AOX). When these cells are switched from a medium containing methanol to one containing glucose, the peroxisomes are selectively sequestered by the vacuole for degradation by a process called micropexophagy (Tuttle and Dunn, 1995). To identify the molecular components of this degradative process, we developed a novel approach whereby genes can be randomly mutagenized *in vivo* by the integration of a vector containing a *zeo^R* gene, pREMI-Z. Those zeocin-resistant cells that were unable to degrade AOX during glucose adaptation were identified by direct colony assay and verified by liquid medium assay (see *Materials and Methods*). Using this approach, we have isolated a number of mutant strains based on their poor ability to degrade AOX during glucose adaptation. The gene mutated by the insertion of the pREMI-Z was sequenced and identified in each of our mutants (see Table 1). We then compared the inability of these mutants to degrade peroxisomes to parental GS115 and to vacuole defective mutants that lack proteinases A (*pep4*) and B (*prb1*) (Figure 1A). Within 6 h of glucose adaptation, more than 90% of the AOX was degraded by the GS115 cells. In comparison, only 10% of the AOX was degraded in mutants lacking proteinases A and B. Less than 60% of the AOX was degraded in R19 cells that contained a pREMI-Z insertion within the open reading frame of the *PpATG9* gene. AOX degradation in these R19 cells was comparable to that observed for *PpAtg1*, *PpAtg2*, and *PpAtg11* mutants, but higher than the 10–30% degraded by *PpAtg7*, *PpAtg18*, *Ppops15*, or *Ppvac8* mutants. Next, we examined the ability of R19 cells to degrade AOX during ethanol adaptation (Figure 1B). We observed on Western blots a substantial loss of AOX protein when GS115 cells adapt from methanol to ethanol medium. However, no loss of AOX was detected in R19 cells over 24 h, suggesting that macropexophagy was suppressed in the cells lacking *PpAtg9*.

Our previous studies have shown that a number of those REMI mutants defective in pexophagy were also defective in nitrogen starvation-enhanced proteolysis (Yuan *et al.*, 1999; Guan *et al.*, 2001; Strømhaug *et al.*, 2001). Therefore, we next quantified protein degradation in R19 starved for amino acids (Figure 1C). When GS115 parental cells are starved for nitrogen and amino acids, the cellular protein was degraded at a rate of 0.36% per hour. The rate of protein degradation in starved cells was significantly lower in cells lacking proteinases A and B, consistent with this degradation being mediated by the vacuole. In cells lacking *PpAtg11* or *Vac8*, cellular protein degradation was 70–90% of control, suggesting these proteins have a minimal role in starvation-induced autophagy. To the contrary, we determined that the degradation of cellular proteins was reduced by 60% in *PpAtg9*

Figure 1. Pexophagy and autophagy are defective in *Ppatg9* mutants. (A) Wild-type GS115, R12 (*Ppatg1*), R22 (*Ppatg2-2*), WDY7 (*Ppatg7*), R19 (*Ppatg9*), R13 (*Ppatg11-1*), R9 (*Ppatg18*), *Ppvps15Δ* (*Ppvps15Δ*), WDY53 (*Ppvac8Δ*), and SMD1163 (*pep4, prb1*) cells were grown in YNM for 36 h. At that time, cells were switched to a medium containing 2% glucose. Aliquots were removed at 0 and 6 h of adaptation, the cells were lysed, and AOX activities were measured as describe in *Materials and Methods*. The data are expressed as a percentage of AOX remaining at 6 h relative to 0 h and represents the mean $\pm$ SE of 3–6 trials. (B) Wild-type GS115 and R19 (*Ppatg9*) cells were grown in YNM for 36 h and then switched to YNE medium. Aliquots were removed, the cells were lysed, and AOX protein was visualized by Western blotting as describe in *Materials and Methods*. (C) Wild-type GS115, R12 (*Ppatg1*), R22 (*Ppatg2-2*), WDY7 (*Ppatg7*), R19 (*Ppatg9*), R13 (*Ppatg11-1*), R9 (*Ppatg18*), *Ppvps15Δ* (*Ppvps15Δ*), WDY53 (*Ppvac8Δ*), and SMD1163 (*pep4, prb1*) cells were grown in minimal medium containing ^{14}C -valine for 18 h. The cells were pelleted and resuspended in medium lacking amino acids and nitrogen and containing 10 mM valine. The production of TCA-soluble radioactivity was measured at 2, 5, 8, and 24 h of chase, and the rates calculated by linear regression of the slope of the line. The rates represent the mean $\pm$ SE of 5–7 trials.

cells starved for amino acids and nitrogen. This value was comparable to the rates of degradation observed in *Ppatg1*, *Ppatg2*, *Ppatg7*, *Ppatg18*, and *Ppvps15* mutants.

PpAtg9 Is Essential for a Sequestration Event in Pexophagy

We have shown that during glucose-induced micropexophagy the vacuole indents while sequestering membranes encircle multiple peroxisomes (Tuttle and Dunn, 1995). The sequestering membranes are labeled with FM 4-64, a dye that selectively labels vacuole membranes, suggesting that they likely form from the vacuole. A micropexophagic membrane apparatus (MIPA) containing PpAtg8 forms near the tips of the sequestering membranes. The MIPA is thought to assist in the fusion of the sequestering membranes, thereby incorporating the peroxisomes into the vacuole for degradation. During ethanol-induced macropexophagy, individual peroxisomes are selectively incorporated into pexophagosomes whose membranes contain PpAtg8. The pexophagosome then fuses with the vacuole and its contents degraded. To better assess the site of blockage of pexophagy in the R19 cells, we examined the formation of the sequestering membranes and the MIPA during micropexophagy and of the pexophagosome during macropexophagy. GS115 (Figure 2, A and C) and R19 (Figure 2, E, G, I, and K) cells were grown in methanol-enriched YNM medium and then adapted to glucose-enriched YND (Figure 2, E and I) or ethanol-enriched YNE (Figure 2, G and K) medium. Cells were harvested at 3 h, fixed in potassium permanganate, and prepared for electron microscopy. During glucose adaptation, profiles of peroxisomes engulfed by the vacuole were routinely observed in GS115 cells (Figure 2A). However, when R19 cells were adapted to glucose, the sequestering membranes appear to be absent, but instead the vacuole appeared only slightly indented (Figure 2E), with an occasional short armlike projection (Figure 2I). Similar vacuole morphology has been reported for *Ppatg11*, *Ppatg18*, and *Ppvps15* mutants (Stasyk *et al.*, 1999; Guan *et al.*, 2001; Kim *et al.*, 2001). Next, we examined whether the MIPA was forming in R19 cells. WDY70 cells expressing BFP-SKL and GFP-PpAtg8 were adapted from YNM to YND for 2 h. At this time, PpAtg8 was localized to the MIPA and foci adjacent to the peroxisomes (Figure 2B). In the absence of PpAtg9, the MIPA was absent and PpAtg8 localized solely to foci (Figure 2, F and J). During ethanol adaptation, peroxisomes within pexophagosomes were observed by electron (Figure 2C) and fluorescence (Figure 2D) microscopy. In WDY70 cells, the PpAtg8 was localized almost exclusively to pexophagosomes (Figure 2D). Pexophagosomes were absent in cells lacking PpAtg9 (Figure 2, G, H, K, and L), and PpAtg8 was localized to multiple foci that appear within and about the peroxisome cluster (Figure 2, H and L). The data demonstrate that PpAtg9 is essential for the formation of sequestering membranes that arise from the vacuole and the assembly of the MIPA during micropexophagy and of the pexophagosome during macropexophagy.

Cellular Localization of PpAtg9 in Growing Cells

Next, we examined the cellular distribution of PpAtg9 in cells grown in glucose medium. This was done by constructing the following expression vectors: GFP-PpAtg9 behind the GAPDH promoter in pIB2 (pTC1), PpAtg9-GFP behind the endogenous PpAtg9 promoter (pTC2), GFP-PpAtg9 behind the GAPDH promoter in pGAPz (pWD17), and mRFP-PpAtg9 behind the GAPDH promoter in pGAPz (pAJM6). PpAtg9-GFP (unpublished data), GFP-PpAtg9 (see Figure 8), and mRFP-PpAtg9 (see Figure 8) were functional as

Figure 2. Pexophagy is blocked at an early sequestration event in cells lacking PpAtg9. Wild-type cells, GS115 (A and C) and WDY70 (B and D) and cells lacking PpAtg9, R19 (E, G, I, and K) and WDY71 (F, H, J, and L) were grown in YNM for 24–36 h. At that time, cells were switched to medium containing 2% glucose (A, B, E, F, I, and J) or 0.5% ethanol (C, D, G, H, K, and L) for 3 h. Cells were fixed with potassium permanganate and prepared for viewing on a JEOL 100CX transmission electron microscope (A, C, E, G, I, and K) or viewed in situ by fluorescence microscopy (B, D, F, H, J, and L). When GS115 and WDY70 cells were adapted to glucose for 3 h, many of the cells lacked peroxisomes, whereas in others the vacuole was found to virtually surround the peroxisome cluster (A and B). In R19 and WDY71 cells, peroxisome clusters were evident in virtually all the cells. The vacuole was either oblong with a cuplike depression at the surface adjacent to the peroxisomes (E and F) or round with short armlike segments (I and J). By fluorescence microscopy, the vacuole was observed by staining with FM 4-64, the peroxisomes by expressing BFP-SKL, and the MIPA and pexophagosomes by expressing GFP-PpAtg8. GFP-PpAtg8 was observed at the MIPA in WDY70 cells (B, arrow), but appeared as foci in WDY71 cells lacking PpAtg9 (F and J, arrowheads). During ethanol adaptation, individual peroxisomes were sequestered into pexophagosomes (C, arrow) that contained PpAtg8 (D, arrow). No pexophagosomes were evident in R19 and WDY71 cells lacking PpAtg9 (G, H, K, and L). In the absence of macropexophagy, PpAtg8 resided in numerous foci (H and L, arrowheads). N, nucleus; P, peroxisome; V, vacuole.

determined by their ability to rescue R19 cells. When these cells were grown in YPD, GFP-PpAtg9 (Figure 3A) localized to one or more foci or structures distributed about the cell periphery. To better define the nature of these structures, we coexpressed GFP-PpAtg9 with DsRed-HDEL and mRFP-PpAtg9 with CoxIV-GFP in GS115 cells. Some structures containing GFP-PpAtg9 appeared to be in close association with endoplasmic reticulum identified by DsRed-HDEL (Figure 3C, arrow) and mitochondria identified by CoxIV-GFP (Figure 3D, arrow). We next examined whether these structures contained Sec13 (intermediate compartment) or Sec7 (Golgi apparatus) by coexpressing mRFP-PpAtg9 with Sec13-GFP and Sec7-GFPx3. The results demonstrate that PpAtg9 does not colocalize with either Sec13 (Figure 3E) or Sec7 (Figure 3F). In *S. cerevisiae*, the peripheral vesicles containing ScAtg9 do not colocalize with any defined organelle markers for the Golgi apparatus, endosomes, or the endoplasmic reticulum (Noda *et al.*, 2000; Kim *et al.*, 2002; Reggiori *et al.*, 2004). Therefore, we will refer to these unique peripheral structures collectively as the Atg9 peripheral compartment (Atg9-PC).

Cellular Trafficking of PpAtg9 during Glucose-induced Pexophagy

The trafficking of PpAtg9 during glucose-induced pexophagy was visualized in TC3 cells expressing BFP-SKL behind the AOX promoter and GFP-PpAtg9 behind the GAPDH promoter (Figure 4). When TC3 cells were grown in YNM, GFP-PpAtg9 localized to one or more structures, described above as the Atg9-PC (Figure 4A). On adapting these cells from YNM to YND for 3 h, GFP-PpAtg9 localized to multiple structures of differing sizes and shapes that were positioned at the vacuole surface labeled with FM 4-64 and to the sequestering membranes that could be seen flanking the peroxisomes labeled with BFP-SKL (Figure 4). We refer

these structures that appear as dots and patches as perivacuolar structures (PVS). The PVS were routinely observed near those sites where the sequestering membranes joined the vacuole, suggesting they may function in the formation of these membranes from the vacuole. Indeed, the sequestering membranes contained both PpAtg11 and PpAtg18 present at the vacuole membrane and stain with FM 4-64, suggesting that they originated from the vacuole (Guan *et al.*, 2001; Kim *et al.*, 2001). These results were not due to overexpression of GFP-PpAtg9 that may be caused by the GAPDH promoter, because PpAtg9-GFP whose expression is regulated by the endogenous PpAtg9 promoter in TC14 cells showed a similar distribution but weaker signal (unpublished data). In a given population of cells, the onset of micropexophagy is variable resulting in cells at differing stages of micropexophagy. Nevertheless, we have presented a time course based on images obtained from 0–2 h of glucose adaptation that best illustrates our understanding of PpAtg9 trafficking (Figure 4C). We show that during glucose-induced pexophagy, PpAtg9 traffics from the Atg9-PC (arrowheads) to the PVS (arrows) and then to the vacuole membrane and those sequestering membranes (double arrowheads) that engulf the peroxisomes for degradation within the vacuole.

We next examined the expression of PpAtg9 in cells during glucose-induced micropexophagy. This was done by constructing a PpAtg9 protein tagged at the N-terminus with an HA epitope. This construct along with the PpAtg9 promoter of ~400 base pairs upstream of the start codon was inserted into the pIB1 vector (Sears *et al.*, 1998). The resulting vector was used to transform GS115 cells and the expression evaluated on Western blots using antibodies that recognized the HA tag. A single protein ~100 kDa was detected in cells expressing HA-PpAtg9. This protein was absent in extracts of GS115 cells that were not transformed (unpublished data). At 4–6 h of glucose-induced pexoph-

Figure 3. Cellular localization of PpAtg9. TC14 expressing PpAtg9-GFP behind the endogenous PpAtg9 promoter (A and B), AJM27 expressing GFP-PpAtg9 behind the GAPDH promoter and dsRFP-HDEL (C), AJM44 expressing mRFP-Atg9 behind the GAPDH promoter and CoxIV-GFP (D), AJM19 expressing mRFP-PpAtg9 behind the GAPDH promoter and Sec13-GFP (E), and AJM18 expressing mRFP-PpAtg9 behind the GAPDH promoter and Sec7-GFP (F) were grown in YPD and the cellular distribution of the GFP- and mRFP-tagged proteins were visualized by in situ fluorescence microscopy. In TC14 cells, PpAtg9-GFP was present in foci situated at the cell periphery (arrows). The GFP-PpAtg9 structures appeared to be juxtaposed to the endoplasmic reticulum containing dsRFP-HDEL (C) and mitochondria containing CoxIV-GFP (D), but distinct from intermediate vesicles containing Sec13-GFP (E) and Golgi apparatus containing Sec7-GFP (F).

agy, the cellular levels of HA-PpAtg9 increased more than twofold relative to 0 h (Figure 4D). Over the next 2 h, the cellular levels of HA-PpAtg9 diminished. Similar results were observed when HA-PpAtg9 was expressed in R19 cells (unpublished data).

Cellular Trafficking of PpAtg9 during Glucose-induced Pexophagy Requires other PpAtg Proteins

Many Atg proteins interact with each other, thereby influencing function and trafficking (Wang and Klionsky, 2003; Reggiori *et al.*, 2004). In fact, the trafficking of ScAtg9 appears to be regulated by a number of ScAtg proteins, including ScAtg1 and ScAtg18 (Reggiori *et al.*, 2004). Therefore, we investigated whether other PpAtg proteins were required for the trafficking of PpAtg9 from the Atg9-PC to the PVS and sequestering membranes. We examined the trafficking of GFP-PpAtg9 during glucose-induced pexophagy in mutants defective in early (*Ppatg11*, *Ppatg18*, and *Ppvps15Δ*), intermediate (*Ppatg2* and *Ppatg7*), and late (*Ppatg1* and *Ppvac8Δ*) sequestration events. These mutants were transformed by electroporation with pTC1 and GFP-PpAtg9 expression verified by Western blotting and fluorescence microscopy. When the resulting transformants were grown in YNM, GFP-PpAtg9 was found almost exclusively at the Atg9-PC (unpublished data). After growth in YNM, the mutants expressing GFP-PpAtg9 were then adapted to glu-

cose for 3 h. In *Ppatg11*, *Ppatg18*, and *Ppvps15Δ* mutants, the vacuoles were predominantly round with a slight indentation and no armlike extensions were evident (Figure 5). GFP-PpAtg9 expressed in *Ppatg11* and *Ppvps15Δ* cells was found predominantly at peripheral foci (Figure 5). In addition, the vacuole membrane was void of GFP-PpAtg9 in these mutants. In *Ppatg18* cells, GFP-PpAtg9 localized to the PVS and to the vacuole membrane (Figure 5). The vacuoles in *Ppatg7* and *Ppatg2* mutants were indented with short extensions (Figure 6). In these cells, GFP-PpAtg9 was found in peripheral structures (arrowheads) and the PVS (arrows) located either at the vacuole or at the site where the sequestering membranes join the vacuole (Figure 6). Although the location of the PVS appeared unaltered, the PVS morphology appeared to be smaller and less diffuse in these mutants than that observed in control TC3 cells (see Figure 4). Furthermore, GFP-PpAtg9 was not detected at the sequestering or vacuole membranes. During micropexophagy, the vacuoles in *Ppatg1* and *Ppvac8Δ* mutants contained an extensive segmented array of sequestering membranes that extended from the vacuole to almost completely surround the peroxisome cluster (Figure 7). GFP-PpAtg9 was found at the PVS and sequestering membranes (Figure 7).

Domains of PpAtg9 Required for Function and Trafficking

We have demonstrated that PpAtg9 traffics from the Atg9-PC to the PVS where it appears to function in the formation of the sequestering membranes. To define those domains that are essential for PpAtg9 function, we have characterized the function and trafficking of PpAtg9 mutants lacking specific peptide regions. We first deleted the N-terminus (M1-Y221), a region of low homology when compared with its *S. cerevisiae* counterpart. Unlike wild-type GFP-PpAtg9, GFP-Ppatg9ΔN only partially rescued R19, suggesting this protein has limited function for glucose-induced micropexophagy (Figure 8A). However, during glucose adaptation, GFP-Ppatg9ΔN could be observed at the Atg9-PC (Figure 8B, arrowheads), PVS (Figure 8B, arrows), and vacuolar membranes, suggesting that the N-terminus is not essential for PpAtg9 trafficking. Next, we deleted a highly conserved loop (T471-A523) between two putative transmembrane domains. As seen for Ppatg9ΔN, Ppatg9ΔL3 was also unable to efficiently rescue the R19 phenotype (Figure 8A). However, unlike Ppatg9ΔN, Ppatg9ΔL3 was not present at the Atg9-PC, PVS, or vacuole membrane but to a compartment morphologically similar to the endoplasmic reticulum (Figure 8C, large arrows). The data suggest that loop T471-A523 is essential for trafficking of PpAtg9 to the Atg9-PC.

Perivacuolar Structures Contain PpAtg9 and PpAtg11, but not PpAtg2

We have previously shown that Atg2 becomes associated with peripheral structures during pexophagy and that this association requires PpAtg9 (Strømhaug *et al.*, 2001). In addition, we have reported that PpAtg11 localizes to the vacuole membrane and to one or more structures associated with either the vacuole or the armlike extensions that surround the peroxisomes (Kim *et al.*, 2001). In this study, we have shown that the trafficking of PpAtg9 requires PpAtg2 and PpAtg11. Therefore, we compared the localization of mRFP-PpAtg9 with PpAtg2-GFP and PpAtg11-GFP in cells adapting from YNM to YND (Figure 9). We first compared the localization of mRFP-PpAtg9 with PpAtg18-GFP, which we have previously shown to be present at the vacuole and

Figure 4. PpAtg9 relocates from peripheral structures to the sequestering membranes during glucose-induced pexophagy. TC3 cells expressing both BFP-SKL behind the AOX1 promoter and GFP-PpAtg9 behind the GAPDH promoter were grown in YNM medium in the presence of FM 4-64, adapted to YND medium for 0 h (A) and 3 h (B), and the cellular distribution of GFP-PpAtg9 visualized by in situ fluorescence microscopy. Peroxisomes were identified by the presence of BFP, which was targeted by its SKL signal and the vacuole by the red dye FM 4-64. At 0 h, many cells had BFP-containing peroxisomes and a single round vacuole. GFP-PpAtg9 was localized to foci near the cell periphery (arrowheads). At 3 h of glucose adaptation, numerous profiles of vacuoles with armlike extensions surrounding the BFP-containing peroxisomes could be observed. GFP-PpAtg9 was present at perivacuolar structures (arrows) and at the vacuole membrane (double arrowhead). In C, images from 0 to 2 h of glucose adaptation depict the movements of GFP-PpAtg9 from peripheral structures (arrowheads) to the perivacuolar structures (arrows) and sequestering and vacuole membranes (double arrowhead). In D, ASK2 cells expressing HA-PpAtg9 behind the endogenous PpAtg9 promoter were grown in methanol medium and then adapted to glucose medium. At 0–8 h, equivalent numbers of cells based on the optical density (OD_{600}) of the cultures were solubilized in SDS, and the proteins were separated by SDS-PAGE. HA-PpAtg9 was then visualized by Western blotting using anti-HA antibodies and quantified using a Typhoon laser scanner. The values were normalized to 0 h and presented as the mean $\pm$ SE ($n = 6$).

sequestering membranes. In cells undergoing micropexophagy, mRFP-PpAtg9 was present in the Atg9-PC and at the PVC localized at sites where the sequestering membranes appear to extend from the vacuole (Figure 9, arrows). Unlike GFP-PpAtg9, mRFP-PpAtg9 was not present at the vacuole membrane, but instead was present within the vacuole that was delineated by PpAtg18-GFP and PpAtg11-GFP. One possible explanation is that the mRFP at the N-terminus of PpAtg9 is exposed to vacuolar enzymes and proteolytically removed from PpAtg9. However, the accumulation of mRFP within the vacuole persisted in cells lacking Pep4 or Prb1 (Tomasini and Dunn, unpublished observations). The data reveal that PpAtg2 does not colocalize with the Atg9-PC nor the PVS. However, some of the PpAtg2 vesicles can be found in close proximity to the PVS (see Figure 9, arrows). PpAtg11 localized to the vacuole membrane and the PVS containing PpAtg9 (Figure 9, arrows), but did not colocalize with the peripheral structures of PpAtg9 (Figure 9, arrowhead). The data suggest that the Atg9-PC does not contain PpAtg2 and that the peripheral compartment of PpAtg2 may be unique. Furthermore, we have shown that the PVS contains PpAtg9 and PpAtg11 but not PpAtg2 or PpAtg18.

PpAtg9 Does Not Localize to MIPA or the Pexophagosome

Finally, we examined whether mRFP-PpAtg9 colocalized with GFP-PpAtg8 at the MIPA, at the pexophagosomes, or at the perivacuolar foci referred to as preautophagosome structures (PAS) in *S. cerevisiae*. During glucose-induced micropexophagy, PpAtg8 was present at perivacuolar foci and at the MIPA (arrowheads), which did not contain PpAtg9 (Figure 10A). During ethanol-induced macropexophagy, PpAtg8 was found at perivacuolar foci and at the pexophagosome (arrowheads), whereas PpAtg9 localized to the Atg9-PC (small arrow) and the PVS (large arrow; Figure 10B). PpAtg9 did not colocalize with PpAtg8 and was absent from the pexophagosome. To the contrary, PpAtg8 was not present at the Atg9-PC (Figure 10B, small arrow) or at the PVS (Figure 10, large arrows). The data suggest that PpAtg9 is not a component of the MIPA or the pexophagosome and that the PVS lacks PpAtg8.

DISCUSSION

Autophagy is an avenue for the degradation of proteins and organelles. This process is critical for cell survival during

Figure 5. Trafficking of PpAtg9 in mutants defective in early sequestration events. TC6 (*Ppatg11*), TC9 (*Ppatg18*), and TC11 (*Ppmps15Δ*) mutants expressing GFP-PpAtg9 behind the GAPDH promoter were grown in YNM medium in the presence of FM 4-64 then adapted to YND medium for 3 h and the cellular distribution of GFP-PpAtg9 visualized by in situ fluorescence microscopy. The vacuoles in the *Ppmps15Δ*, *Ppatg11*, and *Ppatg18* mutants were either round, flattened, or slightly indented. GFP-PpAtg9 was present in peripheral structures (arrowheads) in the *Ppmps15Δ* and *Ppatg11* mutants. However, in *Ppatg18* mutants, GFP-PpAtg9 was visualized at the large sometimes diffuse perivacuolar structures (arrows) and the vacuole membrane (double arrowheads).

times of nutrient deprivation. Twenty-seven autophagy genes (ATG) have been identified using yeast models of autophagy (Klionsky *et al.*, 2003). One such model is *P. pastoris*, in which we can follow the molecular events required for the selective sequestration and vacuolar degradation of peroxisomes by a process called pexophagy. When cells adapt from growth in methanol to glucose, the cells respond by activating the degradation of peroxisomes by pexophagy. During glucose-induced pexophagy, sequestering arms extend from the vacuole to engulf multiple peroxisomes. On homotypic membrane fusion of the arms, the peroxisomes are incorporated into autophagic bodies within the vacuole where they are degraded. We have previously shown that PpAtg2 (Gsa11), PpAtg7 (Gsa7), PpAtg11 (Gsa9), and PpAtg18 (Gsa12) are required for the sequestration events of micropexophagy (Yuan *et al.*, 1999; Guan *et al.*, 2001; Kim *et al.*, 2001; Strømhaug *et al.*, 2001). In this study, we show that PpAtg9 is required for an early sequestration event in micropexophagy, possibly the initial formation of the sequestration membranes that extend from the vacuole. In addition, the assembly of MIPA during micropexophagy and the formation of the pexophagosome during macropexophagy does not occur in cells lacking PpAtg9. PpAtg9 is an integral membrane protein with five possible transmembrane domains that traffics to the vacuole membrane becoming a component of the sequestration membranes that appear to arise from the vacuole. PpAtg9 is not essential for cell viability, but appears to be structurally conserved throughout a number of plant, fungi, insect, and mammalian

Figure 6. Trafficking of PpAtg9 in mutants defective in intermediate sequestration events. TC19 (*Ppatg2Δ*) and TC5 (*Ppatg7Δ*) mutants expressing GFP-PpAtg9 behind the GAPDH promoter were grown in YNM medium in the presence of FM 4-64 then adapted to YND medium for 3 h, and the cellular distribution of GFP-PpAtg9 was visualized by in situ fluorescence microscopy. The vacuoles in *Ppatg2Δ* and *Ppatg7Δ* mutants were indented with an occasional short segmented armlike extension. In these mutants, GFP-PpAtg9 localized to peripheral structures (arrowheads) and the PVS at the vacuole surface (arrows), but was absent from the vacuolar membrane. Similar results were obtained in TC8 (*Ppatg2*) and TC4 (*Ppatg7*) cells.

species. PpAtg9 is also necessary for starvation-induced nonselective autophagy in *P. pastoris*, *S. cerevisiae*, and *A. thaliana*, and for the trafficking of Ape1 to the vacuole in *S. cerevisiae* (Noda *et al.*, 2000; Hanaoka *et al.*, 2002).

In *S. cerevisiae*, ScAtg9 resides in two populations of vesicles, one at the cell periphery and a second found adjacent

Figure 7. Trafficking of PpAtg9 in mutants defective in late sequestration events. TC7 (*Ppatg1*) and TC12 (*Ppvac8Δ*) mutants expressing GFP-PpAtg9 behind the GAPDH promoter were grown in YNM medium in the presence of FM 4-64 and then adapted to YND medium for 3 h, and the cellular distribution of GFP-PpAtg9 was visualized by in situ fluorescence microscopy. The vacuoles in *Ppatg1* and *Ppvac8Δ* mutants were indented with multiple "segmented" armlike extensions that surrounded the peroxisome cluster. GFP-PpAtg9 was visualized at the vacuole membrane but appeared to be more concentrated at the sequestering membrane extensions of the vacuole (double arrowheads). In addition, GFP-PpAtg9 was found at the PVS situated at sites of the vacuole surface adjacent to the armlike extensions (arrows).

Figure 8. PpAtg9 domains required for function and trafficking. In A, wild-type GS115, R19 (*Ppatg9*), and R19 cells expressing mRFP-PpAtg9, GFP-PpAtg9, GFP-Ppatg9ΔN (ΔM1-Y221), or GFP-Ppatg9ΔL3 (ΔT471–A523) were grown in YNM medium for 36 h. All proteins were expressed behind the GAPDH promoter. Cells were then switched to glucose medium. Aliquots were removed at 0 and 6 h of adaptation, the cells were lysed, and AOX activities were measured as described in *Materials and Methods*. The data are expressed as a percentage of AOX remaining at 6 h relative to 0 h and represent the mean $\pm$ SE of 4–6 trials. ASK5 (B) and ASK4 (C) cells were grown in YNM for 20 h in the presence of FM 4-64. The cells were then transferred to YND medium for 3 h and then visualized by fluorescence microscopy. GFP-Ppatg9ΔN was found at the Atg9-PC (arrowheads), PVS (arrows), and the vacuolar membranes. GFP-Ppatg9ΔL3 was absent from the Atg9-PC and PVS, but found in a compartment that resembled the endoplasmic reticulum (large arrows).

to the vacuole. These vesicles distribute as a single peak on sucrose gradients, which can be resolved from the vacuole (Pho8), plasma membrane (Pma1), Golgi apparatus (Kex2), endoplasmic reticulum (Sec12), and endosomes (Pep12; Noda *et al.*, 2000). In addition, our results suggest that those vesicles adjacent to the vacuole are not the prevacuolar compartment (Noda *et al.*, 2000). The preautophagosome structure (PAS) appears as a single structure located at the vacuolar surface and is composed of a number of proteins such as ScAtg2 (Apg2), ScAtg8 (Aut7), ScAtg9 (Apg9), ScAtg11 (Cvt9), ScAtg17 (Apg17), ScAtg18 (Cvt18), ScAtg20 (Cvt20), and ScAtg24 (Cvt13) (Suzuki *et al.*, 2001; Huang and Klionsky, 2002; Kim *et al.*, 2002; Nice *et al.*, 2002; Reggiori *et al.*, 2004). The PAS is thought to be responsible for organizing the formation of the autophagosome and transferring ScAtg8 to the autophagosome membrane. However, the appearance of these structures does not change when cells are starved for amino acids, and other components of the PAS such as ScAtg9 do not appear to associate with autophagosomes (Noda *et al.*, 2000; Kim *et al.*, 2002). Those ScAtg9 vesicles not adjacent to the vacuole but more at the cell periphery differ from the PAS in that they do not contain ScAtg8 (Kim *et al.*, 2002; Tucker *et al.*, 2003). In *S. cerevisiae*,

ScAtg9 appears to recycle between the peripheral vesicles and the PAS. Furthermore, the movements of ScAtg9 from the PAS to the peripheral vesicles require ScAtg1 independent of its kinase activity, ScAtg2, ScAtg18, and the PtdIns 3-kinase complex I, which includes ScVps34, ScVps15, and ScAtg14 (Reggiori *et al.*, 2004).

In Figure 11, we have presented a working model for the trafficking of PpAtg9 during glucose-induced pexophagy. In growing cells, PpAtg9 resides in the Atg9 peripheral compartment (Atg9-PC) that consists of one or more structures distributed near the cell periphery. These structures are neither intermediate vesicles nor the Golgi apparatus because they lack PpSec13 and PpSec7, respectively. However, they occasionally contain the endoplasmic reticulum marker, mRFP-HDEL, whereas others are in close association with the endoplasmic reticulum, suggesting these structures may be a subcompartment of the endoplasmic reticulum. Furthermore, Ppatg9ΔL3 is not present at the Atg9-PC, but appears to be associated with the endoplasmic reticulum. However, additional studies are needed to substantiate the relationship between the endoplasmic reticulum and the Atg9-PC. ScAtg9 appears to recycle between the peripheral compartment and the PAS (Reggiori *et al.*, 2004). Such recy-

Figure 9. Perivacuolar structures contain PpAtg9 and PpAtg11. AJM33 expressing GFP-PpAtg18 and mRFP-PpAtg9, AJM25 expressing GFP-PpAtg2 and mRFP-PpAtg9 and AJM32 expressing GFP-PpAtg11 and mRFP-PpAtg9 were grown in YNM medium and then adapted to YND medium for 2 h, and the cellular distribution of GFP- and RFP-tagged proteins was visualized by in situ fluorescence microscopy. PpAtg9 was found at peripheral structures (arrowheads) and PVS (arrows) and within the vacuole delineated by PpAtg18 and PpAtg11. The Atg9-PC did not contain PpAtg2, PpAtg11, or PpAtg18. GFP-PpAtg2 resided in distinct vesicles of which a few were in close association with the PVS. Meanwhile, virtually all the PVS contained both GFP-PpAtg11 and mRFP-PpAtg9 (arrows).

cling was not detected in *P. pastoris*, although we have not analyzed this under the same conditions defined in *S. cerevisiae*. We have also shown that the PpAtg9-PC is sometimes associated with mitochondria. However, the functional significance of this location remains unclear. Nevertheless, based on our findings and those from *S. cerevisiae*, we suggest that the peripheral vesicles containing PpAtg9 are a unique compartment. On the onset of glucose-induced pexophagy, PpAtg9 is recruited from the Atg9-PC to two or more perivacuolar structures (PVS) and then to those membranes that sequester the peroxisomes. The PVS is in many ways similar to the PAS defined in *S. cerevisiae*. For example, both contain PpAtg9 and PpAtg11 (see Figure 10). Ano *et al.* (2005) have shown that similar perivacuolar spots contain PpAtg24. Both PVS and PAS are located at the vacuole and appear to be essential for organizing the formation of sequestering membranes. However, the PVS does not contain PpAtg2 or PpAtg8 and appears to be structurally different from the PAS. There exist multiple foci of PVS of differing sizes situated about the vacuole and the sequestering membranes, whereas the PAS usually consists of a single, well-defined structure at the vacuole. PpAtg17, a component of the PAS in *S. cerevisiae*, resides in a single structure juxtaposed to the vacuole (Ano *et al.*, 2005). PpAtg24 colocalizes with PpAtg17 but is also found at multiple other perivacuolar structures (Ano *et al.*, 2005). PAS has been implicated in the formation of the autophagosome from membranes of unknown origin, but its function remains

Figure 10. PpAtg9 does not colocalize with PpAtg8 structures. WDY75 expressing GFP-PpAtg8 and mRFP-PpAtg9 were grown in YNM and then adapted to glucose or ethanol medium for 2 h, and the cellular distribution of GFP- and RFP-tagged proteins was visualized by in situ fluorescence microscopy. During glucose adaptation (A), mRFP-PpAtg9 was found within the vacuole and at the PVS (large arrows), but was absent from PpAtg8 foci and MIPA (arrowheads). When cells were transferred from methanol to ethanol medium (B), mRFP-PpAtg9 was present within the vacuole and at the peripheral compartment (small arrow) and the PVS (large arrow), but not at the PpAtg8 foci or pexophagosome (arrowheads).

unclear (Suzuki *et al.*, 2001). We have demonstrated that the PVS is situated at sites where the sequestering membranes appear to form from the vacuole and that PpAtg9 is transported from the PVS to those sequestering membranes that engulf the peroxisomes. Therefore, we project that PVS may act as the site of assembly in the formation of the sequestering membranes that appear to originate from the vacuole because they stain with FM 4-64. It is unclear how these membranes are formed. Mukaiyama *et al.* (2002) suggest these membranes form by a "septation" of the vacuole. Our data suggest that the formation of these membranes requires protein synthesis and both PpAtg9 and PpAtg18. We have previously shown that these membranes do not form in the presence of cycloheximide (Tuttle and Dunn, 1995). In this study, we show that the cellular levels of PpAtg9 are increased during pexophagy, suggesting a role for protein synthesis. In addition, we have demonstrated that when PpAtg9 is absent or does not traffic to the PVS, the vacuole arms are either not present or short. Conversely, when PpAtg9 localizes to the PVS and the vacuole membrane but PpAtg18 is missing, the vacuole arms are absent. Furthermore, we have shown that PpAtg9 lacking its N-terminus is sorted to the PVS, but the peroxisomes are not sequestered or degraded. This suggests that there exists a region within the N-terminus of PpAtg9 that is essential for its function once it arrives at the PVS. This domain and its function have yet to be defined and are currently under investigation.

We have shown that PpAtg11 and PpVps15, but not PpAtg18, PpAtg2, PpAtg7, PpAtg1, or PpVac8, are essential to recruit PpAtg9 to the PVS. In *S. cerevisiae*, the overexpression of ScAtg11 appears to enhance the recruitment of ScAtg9 to the PAS (Kim *et al.*, 2002). In cells lacking ScAtg1, ScAtg2, ScAtg14, or ScAtg18, ScAtg9 is found predominantly at the PAS either because trafficking to the PAS is enhanced or recycling to the peripheral vesicles is suppressed (Reggiori *et al.*, 2004). ScAtg11 and ScAtg18 are present at the PAS and vacuole surface, but the localization

Figure 11. Model of PpAtg9 trafficking during pexophagy. During growth in YNM, PpAtg9 (green) is in the peripheral compartment (Atg9-PC), PpAtg11 (red) at the vacuole membrane, and PpAtg2 (brown) cytosolic. Upon glucose-induced pexophagy, PpAtg9 is trafficked from the Atg9-PC to the PVS juxtaposed to the vacuole which also contains PpAtg11, and to the sequestering membranes (SM) that surround the peroxisomes (blue). Also at this time, PpAtg2 becomes associated with unknown structures situated near the PVS. The trafficking of PpAtg9 from the Atg9-PC to the PVS requires PpAtg11 and PpVps15. The movements of PpAtg9 from the PVS to the vacuolar and sequestering membranes require PpAtg7 and PpAtg2. The formation of the sequestering membranes from the vacuole requires PpAtg18, but the movements of PpAtg9 to the vacuolar membrane proceeds normally in the absence of this protein. Finally, the fusion of the sequestering membranes that enable the incorporation of the peroxisomes into the vacuole is guided by the micropexophagy-specific membrane apparatus (MIPA) and requires PpVac8 and PpAtg1.

of ScVps15 has not yet been determined (Guan *et al.*, 2001; Kim *et al.*, 2001). From the PVS, PpAtg9 is transferred to the membranes of the vacuole and those sequestering membranes that extend from the vacuole. The apparent movement of PpAtg9 from the PVS to the sequestering mem-

branes requires PpAtg2 and PpAtg7, but not PpAtg18, PpAtg1, or PpVac8. We have previously shown that during pexophagy PpAtg2 becomes associated with foci of unknown origin (Strømhaug *et al.*, 2001). In this study, we show that these structures are situated juxtaposed to the PVS. The homotypic fusion of the sequestering membranes and formation of the autophagic body within the vacuole completes the sequestration of the peroxisomes. A membrane sac called MIPA (micropexophagy-specific membrane apparatus), which contains PpAtg8 and PpAtg26, forms between the tips of the sequestering membranes to presumably direct membrane fusion (Oku *et al.*, 2003; Mukaiyama *et al.*, 2004). In addition to PpAtg8 and PpAtg26, the assembly of MIPA requires the lipidation of PpAtg8, which is mediated by PpAtg3, PpAtg4, and PpAtg7. We show here that the assembly of MIPA also requires PpAtg9. However, it is unclear whether PpAtg9 has a direct role in MIPA formation or if the assembly of MIPA requires the presence of the sequestering membranes. Our data suggest that late sequestration or fusion events require PpAtg1, a serine-threonine protein kinase, and PpVac8, an Armadillo-repeat protein structurally homologous to ScVac8 that when anchored to the vacuole membrane by palmitoylation will promote homotypic vacuole fusion (Wang *et al.*, 1998). PpAtg24 is also essential for homotypic fusion of the sequestering membranes (Ano *et al.*, 2005). PpAtg24 is not required for MIPA formation (Ano *et al.*, 2005), but it is unclear whether PpAtg1 and PpVac8 are essential for the formation of the MIPA.

In summary, we have characterized the functional role of PpAtg9 in glucose-induced micropexophagy. We have shown that PpAtg9 is essential for the formation of the sequestering membranes that engulf the peroxisomes for degradation within the vacuole. During micropexophagy, PpAtg9 traffics from a unique compartment of the Atg9-PC to the PVS juxtaposed to the vacuole where it then becomes associated with the vacuole and those sequestering membranes that engulf the peroxisomes for degradation (see Figure 11). We have also demonstrated that the trafficking of PpAtg9 requires PpAtg11, PpVps15, PpAtg2, and PpAtg7, but not PpAtg1, PpAtg18, or PpVac8. We propose that upon the onset of micropexophagy, PpAtg11 recruits PpAtg9 to the PVS, which act as sites of formation of the sequestering membranes presumably by causing segmentation of the vacuole. These membranes then engulf the peroxisomes and eventually fuse with the assistance of PpAtg1 and PpVac8 to incorporate the peroxisomes into the vacuole for degradation.

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