GSA11 Encodes a Unique 208-kDa Protein Required for Pexophagy and Autophagy in Pichia pastoris

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Cells are capable of adapting to changes in their environment by synthesizing needed proteins and degrading superfluous ones. Pichia pastoris synthesizes peroxisomal enzymes to grow in methanol medium. Upon adapting from methanol medium to one containing glucose, this yeast rapidly and selectively degrades peroxisomes by an autophagic process referred to as pexophagy. In this study, we have utilized a novel approach to identify genes required for this degradative pathway. Our approach involves the random integration of a vector containing the Zeocin resistance gene into the yeast genome by restriction enzyme-mediated integration. Cells unable to degrade peroxisomes during glucose adaptation were isolated, and the genes that were disrupted by the insertion of the vector were determined by sequencing. By using this approach, we have identified a number of genes required for glucose-induced selective autophagy of peroxisomes (GSA genes). We report here the characterization of Gsa11, a unique 208-kDa protein. We found that this protein is required for glucose-induced pexophagy and starvation-induced autophagy. Gsa11 is a cytosolic protein that becomes associated with one or more structures situated near the vacuole during glucose adaptation. The punctate localization of Gsa11 was not observed in gsa10, gsa12, gsa14, and gsa19 mutants. We have previously shown that Gsa9 appears to relocate from a compartment at the vacuole to engulf the peroxisomes. In the gsa11 mutants, the vacuole partially surrounded the peroxisomes, but Gsa9 was still distributed around the peroxisome cluster. This suggests that Gsa9 binds to the peroxisomes independent of the vacuole. The data also indicate that Gsa11 is not necessary for Gsa9 to interact with peroxisomes but acts at an intermediate event required for the vacuole to engulf the peroxisomes.

These sequestration events can be selective or nonselective. Saccharomyces cerevisiae selectively delivers aminopeptidase I to the vacuole by a pathway analogous to autophagy (3). In addition, mammalian and yeast cells are capable of selectively removing peroxisomes by autophagy when these organelles become superfluous because of nutritional changes (1, 8–13). Nonselective degradation of endogenous proteins and recycling amino acids for protein synthesis by autophagy are vital for cellular survival during nutrient deprivation (14, 15).

We have characterized both selective and nonselective autophagy in the yeast, Pichia pastoris. This methylotrophic yeast is capable of utilizing methanol as a sole source of carbon by synthesizing large amounts of peroxisomal alcohol oxidase and other enzymes required for methanol assimilation. If the peroxisomes are no longer required because of nutritional changes, two morphologically and genetically distinct selective autophagic pathways may be activated for their removal and degradation (9, 16). Upon adaptation from methanol to ethanol, a multilamellar membrane of unknown origin can be observed to surround individual peroxisomes before they are delivered to and degraded by the vacuole. This pathway is analogous to macroautophagy in mammalian cells (1, 17, 18). Upon adaptation from methanol to glucose, the vacuole itself can be seen to engulf the peroxisomes by a process identified as microautophagy because of the similarity to a lysosomal invagination process seen in mammalian cells (19–21). We will use the term pexophagy (micro and macro pathways) in reference to the autophagy of peroxisomes because of its selectivity for degrading these organelles (3, 22, 23). In addition to pexophagy, P. pastoris also displays nonselective autophagy upon nutrient starvation whereby portions of the cytosol are sequestered into autogroupomes (macroautophagy) or directly into the vacuole (microautophagy).

We have identified a number of GSA1 genes that are necessary for glucose-induced selective autophagy of peroxisomes (micropexophagy) in P. pastoris. The S. cerevisiae homologues of many of these genes have been shown to be required for both selective and nonselective autophagy (Table I). The studies from a number of laboratories have resulted in many S. cerevisiae genes having multiple names. For our purposes, we will conform to the names used by the Saccharomyces Genome Database. We have shown that two genes, GSA1 and GSA9, are required for early events of glucose-induced pexophagy but not starvation-induced nonselective autophagy. GSA11 encodes

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¶ The abbreviations used are: GSA, glucose-induced selective autophagy; AOX, alcohol oxidase; FM 4-64, N-(triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide; GPP, green fluorescent protein; BFP, blue fluorescent protein; REMI, restriction enzyme-mediated integration; PCR, polymerase chain reaction; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SKL, serine, lysine, and leucine.

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These homologues belong to a family of E1 enzymes—workers (30) have shown that to the vacuole during autophagy (28, 29). Veenhuis and co-
sorting of proteins from the Golgi and cytosol to the vacuole
required for multiple transport pathways responsible for the
with Vps34, a phosphoinositol 3-kinase (28). Both proteins are
it has been shown that Aut7 is not required for formation, but
believed that the conjugation of Apg12 to Apg5 is essential for
reaction that is catalyzed by Apg7 and Aut1 (36, 37). It is
found that Gsa10, a protein homologous to the serine/threonine
Apg17, and a vacuolar membrane protein required for vacuole
for selective and nonselective autophagy, Apg1, Apg13, and
somes. Our data suggest that peroxisome recognition by the
compartment, to the vacuolar arms that sequester the peroxi-
sequestration of peroxisomes, possibly by bringing the opposing vac-
ular membranes together for homotypic fusion to take place (32).

Studies have shown that microautophagy and macroautophagy are morphologically distinct pathways for the selective and
nonselective degradation of cellular proteins. However, data from both S. cerevisiae and P. pastoris models suggest that the
sequestration events clearly require many of the same proteins. For example, many of the CVT genes required for selective macroautophagy of aminopeptidase I are analogous to APG and AUT genes required for nonselective macroautophagy (Table I). In addition, data from Mayer’s laboratory (39, 40) reveal that nonselective microautophagy in S. cerevisiae is reduced without Apg1, Apg7, Aut1, or Aut7. We have reported that Gsa7 is required for both microperoxisome and macroautophagy in P. pastoris (32). In addition, we have observed that many of our GSA genes required for microperoxisome (selective micro-autophagy) are also necessary for macroautophagy. Finally, these degradative pathways converge at the vacuole and require vacuolar hydrolytic enzymes such as Pep4, Prb1, and Cvt17 (6, 9, 16, 41). The data indicate that there exist many molecular events common to micro- and macroautophagy.

In the present work, we describe a novel protein required for microperoxisome, macroperoxisome, and starvation-induced au-
tophagy in P. pastoris. Gsa11 is a 208-kDa protein with homolo-
gues in many eukaryotes. The data suggest that Gsa11 is not required for Gsa9 to interact with the peroxisomes but for later event in the sequestration process. However, unlike Gsa9 we have no evidence to suggest that Gsa11 acts at the site of sequestration. Indeed, when cells adapt from methanol to glu-
cose, Gsa11 relocates from the cytosol to structures that are juxtaposed to the vacuole surface opposite to the site of seque-
tration. These structures may be analogous to the Apg9 compart-
ment that we have described in S. cerevisiae and that contains Apg2, the S. cerevisiae homologue of Gsa11 (42). The
localization of Gsa11 to this compartment requires Gsa10, Gsa12, PpVps15, and Gsa14, the P. pastoris homologue of Apg9 but not Gsa9 or Gsa7. We propose that the association of Gsa11 with this compartment is essential for vacuole sequestration of peroxisomes.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—The yeast strains used in this study are listed in Table II 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/liter biotin, and 0.5% methanol) to induce peroxisome biogenesis. The degradation of peroxi-
somes was induced when cells grown in YNM were transferred to YND (0.67% yeast nitrogen base, 0.4 mg/liter biotin, and 2% glucose). Nitro-
gen starvation medium contained 0.17% yeast nitrogen base (without amino acids and NH₄SO₄) and 2% glucose. All media contained 2% agar when made as plates. Histidine or arginine or both were added at 0.4 mg/liter when needed. Zeocin was added at 25 μg/ml when culturing
Escherichia coli (DH5α) and 100 μg/ml when culturing P. pastoris.

**Yeast Transformation**—Cells grown overnight in YPD to a density of A₅₀₀ = 1.0 were harvested and treated with 10 nm dithiotreitol 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 DNA and transferred to a 0.2-cm gap cuvette (Bio-Rad), and the DNA was introduced by electroporation at 1.5 kV, 25 microfarads, 400 ohms (Gene Pulser, Bio-Rad Corp.). The cells were transferred to plates containing 0.67% yeast nitrogen base without amino acids, 2% glucose, 1 M sorbitol, 0.4 mg/liter biotin, and 2% agar and incubated at 30 °C for 3–5 days before colonies appeared.

**Isolation of gsa Mutants and Cloning of GSA Genes by Restriction Enzyme-mediated Integration (REMI) Mutagenesis**—The theory behind
our mutagenesis approach is to randomly insert a vector with a select-

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**Table I**

| P. pastoris | S. cerevisiae |
|-------------|--------------|
| GSA gene   | APG² | AUT³ | CVT⁴ | VPS⁵ |
| 1           | 7    | 7    | 10   | 18   |
| 3           | 1    | 1    | 7    | 8    |
| 5           | 2    | 2    | 10   | 18   |
| 6           | 9    | 9    | 15   | 66   |
| 8           | 4    | 4    | 15   | 66   |
| 10          | 2    | 2    | 15   | 66   |
| 12          | 30   | 30   | 29, 70 | 29, 70 |
| 14          | 30   | 30   | 29, 70 | 29, 70 |

² P. E. Strømhaug and W. A. Dunn, Jr., unpublished results.

² Genes required for glucose-induced selective autophagy of peroxi-
somes in P. pastoris.
³ Genes required for starvation-induced autophagy in S. cerevisiae.
⁴ Genes required for starvation-induced autophagy of fatty acid syn-
these in S. cerevisiae.
⁵ Genes required for cytoplasm to vacuole targeting of aminopepti-
dase I in S. cerevisiae.
⁶ Genes required for vacuole protein sorting in S. cerevisiae.
able marker into the genome of *P. pastoris* and then screen for *gsa* mutants caused by disruption of gene expression. Vector integration is assisted by the presence of BamHI or DpnII, which will randomly cleave the genomic DNA in vivo leaving 4-base overhangs that are compatible to the overhangs of a BamHI-linearized vector. *pREMI* (provided by Dr. Ben Glick, University of Chicago) is a plasmid, which contains a Zeocin resistance gene with the TEF promoter from *pPICz* (Invitrogen, San Diego, CA) flanked by 55 base pairs of sequence analogous to the genomic DNA.

Construction of *gsa11Δ*—Forward (5′ TCCACTTTGAGCTCGACT-ATCAACGATCGGGAACAGTATTTACACGACAGTTCTGAGTCAGAGAAAGAT-CGAGACGCTCCGCTTAA3′) and reverse (5′ GCTCCCAAAACTGTTTGGAAGAATGACCGCGAGAAACCTTGCATTCTGAGAAAGAT-CGAGACGCTCCGCTTAA3′) primers were used to PCR-amplify the *Zeocin* resistance gene with the TEF promoter from *pPICz* (Invitrogen, San Diego, CA) flanked by 55 base pairs of sequence analogous to the genomic DNA. The PCR products were gel-purified and used to transform GS115. The transformants were selected on YPD plates containing 1 M sorbitol and 100 μg/ml zeocin and replica-plated to YNM plates. Possible null *gsa11Δ* mutants were identified by direct colony assay (see below). The *gsa11Δ* mutant was then verified by liquid assay (see below) and by PCR using primers flanking *GS11* and within the *Zeocin* resistance gene.

Quantitative Assessment of Alcohol Oxidase (AOX) Degradation by Direct Colony Assay—The direct colony assay to detect peroxisomal AOX was performed as described previously with some modifications (9, 46). Briefly, colonies were replica-plated from Zeocin selection plates onto YNM plates and allowed to grow for 3–4 days. At this time, replicas on nitrocellulose were placed onto YN plates for 12 h. The colonies on the nitrocellulose were frozen in liquid nitrogen for 20 s to lyse the cells. AOX activity was then visualized by placing the nitrocellulose on Whatman paper soaked with 33 mM potassium phosphate buffer, pH 7.5, containing 0.13% methanol, 3.4 units/ml horseradish peroxidase, and 0.53 mg/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) at room temperature for 90–60 min.

Quantitative Assessment of AOX Degradation by Liquid Medium Assay—Cells were grown in 20 ml of YNM with methanol as the sole carbon and energy source. At 40 h, 0.4 g of glucose or 100 μl of ethanol was added. Aliquots (2 ml) of cells (8.0×10^6) were harvested at 0 h and at 6 h of glucose or ethanol adaptation were pelleted and resuspended in 1 ml of 20 mM Tris, pH 7.5, containing 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml pepstatin A, and 0.5 μg/ml leupeptin. The cells were then lysed by vortexing in the presence of 50 μl of glass beads (425–600 μm). The glass beads and cellular debris were removed by centrifugation, and AOX was measured by adding 50 μl of this extract to 3 ml of reaction mix containing 3.4 units/ml horseradish peroxidase and 0.53 mg/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 33 mM potassium phosphate buffer, pH 7.5 (8, 9). The reaction was started by adding 10 μl of methanol and continued at room temperature for 30–60 min. Subsequently, the assay was stopped by adding 200 μl of 4 N HCl and the absorbance read at 410 nm.

| Strain | Genotype | Source |
|--------|----------|--------|
| GS115  | *his4*   |        |
| SMD1163| *his4 pep4 prb1* |        |
| PPF1   | *his4 arg4* |        |
| WDKO1  | *GS115::gsa1Δ: ARG4* |        |
| DMM1   |        |        |
| STW1   | *PFP1 his4: pTW51 (Pox1BFP-SKL, HIS4)* |        |
| WDK7   | *SFP1* |        |
| WDK07  | *PFP1* |        |
| Ppsps15D| *his4 arg4 vps15:: ARG4* |        |
| R2     | *GS115 gsa12:: Zeocin* | This study |
| R8/R5  | *GS115 gsa9:: Zeocin* | This study |
| R12    | *GS115 gsa10:: Zeocin* | This study |
| R11/R15| *GS115:: Zeocin* | This study |
| R19    | *GS115 gsa14:: Zeocin* | This study |
| WDY22  | *STW1:: pPS11 (gsa11:: Zeocin)* | This study |
| WDY36  | *R22 his4:: pSP67 (Pgal::GFPIHA-GSA11, NT, HIS4)* | This study |
| WDY37  | *R22 his4:: pSP69 (Pgal::GFPIHA-GSA11, HIS4)* | This study |
| WDY38  | *R2 his4:: pWD12 (Pgal::GFPIHA-GSA11, CT, HIS4)* | This study |
| WDY40  | *DM11 his4:: pSP69 (Pgal::GFPIHA-GSA11, HIS4)* | This study |
| WDY46  | *R2 his4:: pSP69 (Pgal::GFPIHA-GSA11, HIS4)* | This study |
| WDY47  | *R8 his4:: pSP69 (Pgal::GFPH-A-GSA11, HIS4)* | This study |
| WDY48  | *R9 his4:: pSP69 (Pgal::GFPH-A-GSA11, HIS4)* | This study |
| WDY49  | *R12 his4:: pSP69 (Pgal::GFPH-A-GSA11, HIS4)* | This study |
| WDY50  | *WDY7 his4:: pSP69 (Pgal::GFPH-A-GSA11, HIS4)* | This study |
| WDY51  | *Ppsps15D his4:: pSP69 (Pgal::GFPH-A-GSA11, HIS4)* | This study |
| ANB6   | *WDY7 his4:: pSS64 (Pgal::GFPH-A-GSA9, HIS4)* | This study |
| ANB7   | *R2 his4:: pSS64 (Pgal::GFPH-A-GSA9, HIS4)* | Guan et al.5 |
| ANB9   | *R11 his4:: pSS64 (Pgal::GFPH-A-GSA9, HIS4)* | Guan et al.5 |
| ANB12  | *WDK09 his4:: pSS64 (Pgal::GFPH-A-GSA9, HIS4)* | Guan et al.5 |

3 Guan, J., Stremhaug, P. E., George, M. D., Habibzadeh-Tari, P., Bevan, A., Dunn, W. A., Jr., and Klionsky, D. J. (2001) Mol. Biol. Cell, in press.
**RESULTS**

gsa11 Mutants Are Defective in Selective Pexophagy and Non-selective Autophagy—When *P. pastoris* is grown on methanol as the sole carbon and energy source, it responds by synthesizing peroxisomal enzymes such as AOX required for assimilation of this compound. Afterward, when the medium is changed to glucose or ethanol, these enzymes, which are no longer required for survival, are rapidly degraded. The metabolic shift from methanol to ethanol induces macroperoxisome, whereas the shift from methanol to ethanol induces macroperoxisome (1, 9). To identify components required for these processes, we developed a novel approach that allows us to disrupt gene expression and rapidly identify GSA genes required for glucose-induced selective autophagy (i.e. microperoxisome). We transformed GS115 cells by electroporation with BamHI (or DpnII) restriction enzyme and pREMI linearized by BamHI. The integration of pREMI, a vector containing a Zeocin resistance gene, into the genome was enhanced by the random BamHI cuts, which yields 4-base overhangs compatible to those at the ends of the linearized pREMI. Those mutants, unable to degrade AOX during glucose adaptation, were identified by direct colony assay and verified by liquid medium assay. This procedure resulted in several mutants defective in peroxisome degradation upon glucose adaptation. Three of these mutants (R11, R15, and R22) had the pREMI inserted into the open reading frame of the same gene, GSA11. Southern blots were done to verify that the pREMI vector inserted into a single gene locus in each of these mutants (data not shown). R11 and R15 were proved by sequencing to be the same mutant and were designated gsa11-1, whereas R22 will be referred to as gsa11-2.

The degradation of AOX during glucose adaptation was severely impaired in both gsa11-1 and gsa11-2 strains (Fig. 1A). At 6 h, 5-fold more AOX activity remained in these mutants than was observed in control GS115 cells. The degradation of AOX during ethanol adaptation was also impaired (Fig. 1B). However, the amounts of AOX remaining after 6 h of glucose or ethanol adaptation were substantially lower than that observed in SMD1163 (pep4 prb1) cells, suggesting that gsa11-1 and gsa11-2 cells do not harbor a complete block in microperoxisome or macroperoxisome. Next, we examined the ability of these mutants to degrade endogenous proteins during nitrogen starvation. In yeast, nitrogen starvation induces the nonsselective delivery of cellular components to the vacuole by microautophagy and macroautophagy (3, 4, 39). Starvation-induced proteolysis was significantly inhibited in gsa11 mutants when compared with control cells (Fig. 1C). The suppression of proteolysis was virtually complete, being comparable with that observed in vacuolar hydrolyse-defective SMD1163 cells. The data suggest that Gsa11 is an essential component shared by selective and nonsselective micro- and macroautophagy in *P. pastoris*.
resent the mean calculated by linear regression of the slope of the line. The rates represented activity was measured at 2, 5, 8, and 24 h of chase, and the rates were

\[ \frac{\text{H}}{11006} \]

\[ \frac{\text{H}}{9004} \]

\( \text{gsa11} \)

\( \text{pep4} \)

\( \text{gsa11} \)

\( \text{SMD1163} \)

\( \text{pep4} \)

\( \text{H}9004 \)

S.D. of 3

- adaptation, the cells lysed, and AOX activities measured as described

\( \text{BA} \)

\( \text{A} \)

\( \text{agy} \).

\( \text{FIG. 1} \).

\( \text{gsa11} \) mutants are defective in pexophagy and autophagy. A and B, wild type GS115, R11 (gsa11-1), R22 (gsa11-2), WDRO11 (gsa11Δ), and SMD1163 (pep4, prb1) cells were grown in YNM for 36 h. At that time, cells were switched to medium containing either 2% glucose (A) or 0.5% ethanol (B). Aliquots were removed at 0 and 6 h of adaptation, the cells lysed, and AOX activities measured as described under “Experimental Procedures.” The data are expressed as a percentage of AOX remaining at 6 h relative to 0 h and represent the mean ± S.D. of 3–6 trials. C, wild type GS115, R22 (gsa11-2), WDRO11 (gsa11Δ), and SMD1163 (pep4, prb1) cells were grown in minimal medium containing \( [\text{14C}] \) 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 trichloroacetic acid-soluble radioactivity was measured at 2, 5, 8, and 24 h of chase, and the rates were calculated by linear regression of the slope of the line. The rates represent the mean ± S.D. of 3–5 trials.

These projections meet and fuse thereby fully enclosing the peroxisomes inside the vacuole. Finally, the degradation of the limiting membrane of the intravacuolar vesicle that contains the peroxisomes allows the hydrolysis of the peroxisome and its enzymes. We have been able to classify many of our mutants by comparing vacuole morphologies during micropexophagy. For example, we have previously shown that in the gsa1 mutant the vacuole remains round, consistent with Gsa1 being required for an early event of peroxisome sequestration, whereas in the gsa7 mutant the vacuole surrounds the peroxisomes but fails to complete sequestration consistent with Gsa7 being required for a late sequestration event (32, 46).

To examine vacuole movements around peroxisomes in situ, we constructed a gsa11 mutant that expresses GFP with an SKL (serine, lysine, and leucine) peroxisomal targeting signal at its C terminus. The pREMI vector with flanking DNA of GSA11 isolated from the R11 mutant was used to transform STW1 cells, which express GFP-SKL when grown in methanol. The resulting cells (WDY22; gsa11-1) were defective in degrading AOX during glucose adaptation (data not shown). WDY22 cells were grown in methanol and labeled with FM 4-64, a fluorescent dye that is taken up by endocytosis and stains the vacuolar membrane (49). The cells were then adapted to glucose medium for 3 h and the fluorescence was examined immediately. We observed that the vacuole had a cup-like appearance and only partially surrounded the peroxisomes (Fig. 2A).

Next, gsa11-2 cells were transferred from methanol medium to glucose for 3 and 5 h at which time the cells were fixed with permanganate and processed for electron microscopy. At 3 and 5 h, slightly indented vacuoles were observed adjacent to clusters of peroxisomes (Fig. 2, B and C). Occasionally, short finger-like projections of the vacuole can be seen to extend partially around the peroxisomes (arrows, Fig. 2). This was quite different from the round vacuoles observed in the gsa1 mutant (46) and from the vacuolar extensions that almost completely surround the peroxisomes in the gsa7 mutant (32). The data suggest that micropexophagy was suppressed at an intermediate stage of the sequestration process when Gsa11 is absent. Finally, we examined the morphology of the gsa11 mutant undergoing adaptation from methanol to ethanol (i.e. macropexophagy). We have reported previously that during ethanol adaptation each peroxisome is incorporated into an autophagosome bound by two or more membranes (9). Afterward, the autophagosome fuses with the vacuole, and the peroxisome is degraded. At 3 h of ethanol adaptation, multiple sequestering membranes were not observed around or in association with peroxisomes (Fig. 2D). This suggests that the gsa11 mutant was blocked at an early event in macropexophagy. The data suggest that Gsa11 is important for an event that is common to vacuole and autophagosome sequestration of peroxisomes.

GSA11 Encodes a Unique Protein of 208 kDa—The completed GSA11 sequence revealed a large gene encoding 1862 amino acids of 208 kDa with homology to S. cerevisiae YNL242w (SP072). We have shown recently that YNL242w complements apg2, a mutant defective in starvation-induced autophagy (42). In addition, we have shown that the cytoplasmic to vacuole targeting of aminopeptidase I is defective in this mutant. Structural homologues of Gsa11 also exist in Schizosaccharomyces pombe (Entrez Protein accession number T40198), Drosophila melanogaster (AAF47687), Caenorhabditis elegans (T16637), and in humans (T90051). The similarity is particularly high at the N terminus (1–80 residues) and C terminus (1045–1862 residues), whereas the central region has limited homology (Fig. 3). Although this protein is rather large, it has few recognizable motifs. A putative leucine zipper at amino acids 1455–1476 of Gsa11 appears to be conserved in Apg2, but an ATP/GTP-binding site at amino acids 1517–1524 of Gsa11 is not present in Apg2. There also exists a zinc finger domain at 631–659 residues within the central region of Gsa11. In addition, Gsa11 has multiple serine-rich domains (residues 243–295, 671–684, and 1318–1339) that do not appear to be conserved in Apg2. We have determined by sequencing that the pREMI vector is inserted between valine 1197 and proline 1198 in the gsa11-1 mutant and between aspartic acid 1339 and isoleucine 1340 in the gsa11-2 mutant. In both cases, the conserved region of the C terminus of Gsa11 was disrupted.
We next constructed a null mutant of gsa11. The Zeocin resistance gene was inserted into the GSA11 gene thereby deleting the upstream 84 base pairs and 2903 base pairs of the coding region. The insertion that eliminated the start codon over 950 amino acids was verified by PCR analyses (data not shown). Similar to that seen for the gsa11 mutants, gsa11Δ cells were unable to degrade AOX during glucose (Fig. 1A) or ethanol (Fig. 1B) adaptation. In addition, these cells were defective in starvation-induced protein degradation (Fig. 1C). The similarities in the phenotypes among gsa11-1, gsa11-2, and gsa11Δ cells suggest that the truncated forms of GSA11, presumably expressed in the R11/R15 (gsa11-1) and R22 (gsa11-2) mutants, were completely inactive.

Gsa11 Localizes to Cytoplasmic Structures That Are Proximal to the Vacuole—To better understand how Gsa11 functions in pexophagy and autophagy, we next examined the cellular distribution of Gsa11. This was done by first constructing a GFP recombinant of GSA11 whereby GFP is fused to the N terminus of GSA11. This gene thereby GFP/HA-Gsa11 was predominantly cytosolic. However, at 2 h of adaptation (Fig. 4B, Glucose), GFP/HA-Gsa11 localized to one or more cytoplasmic structures that were close to but not necessarily at the vacuole surface (arrows, Fig. 4B). These structures may correspond to those containing Apg2 (Gsa11 homologue) and Apg9 (Gsa14 homologue) in S. cerevisiae (42). In addition, these structures appeared to be near the vacuolar surface opposite to the site of peroxisome sequestration. Localization of Gsa11 to the vacuolar membrane or sequestering arms was not observed. The association of Gsa11 with this compartment was not an artifact of overexpression due to the GAPDH promoter. Indeed, the association of Gsa11 with these structures was dependent upon several proteins. That is, GFP/HA-Gsa11 remained cytosolic in several gsa mutants despite similar growth conditions (see below). The correlation of the onset of microperoxophagy with the association of Gsa11 with one or more of these structures, which we refer to as the perivacuolar compartment, suggests that this compartment may be the functional site for Gsa11.

The C Terminus Is Required for Gsa11 Function and Its Localization to the Perivacuolar Compartment—There exists two primary regions of similarity between Gsa11 with Apg2 at the N terminus and at the C terminus (Fig. 3). Therefore, we constructed two truncated forms of GSA11, both fused via the N terminus to GFP and an HA epitope. The N-terminal truncation was missing 167 amino acids, whereas the C-terminal truncation was missing 625 amino acids. The full-length Gsa11 and the N-terminal truncated Gsa11(NT) proved to be functional in glucose-induced pexophagy, i.e., gsa11 cells expressing either GFP/HA-Gsa11 or GFP/HA-Gsa11(NT) were found to

![Image](http://www.jbc.org/)

**FIG. 2.** An intermediate event of microperoxophagy and an early event in macroperoxophagy are blocked in gsa11 mutants. WDY22 (gsa11-1, PAOX, GFP/SKL) (A), and R22 (gsa11-2) (B-D) cells were grown in YNM for 36 h. At that time, cells were switched to medium containing either 2% glucose for 3A or 5 h (B) or 0.5% ethanol for 3 h (D). The morphology of the peroxisomes and vacuoles was observed by fluorescence microscopy (A) and by electron microscopy (B–D). The peroxisomes containing GFP-SKL and the vacuoles labeled with the red dye, FM 4-64, were visualized in situ by fluorescence microscopy. Cells were also fixed in potassium permanganate and prepared for viewing on a JEOL 100CX transmission electron microscope (9, 32). During glucose adaptation, peroxisomes were found outside the vacuole of gsa11-1 (A) and gsa11-2 (B and C) mutants. The vacuoles in these mutants formed a cup-like structure with arm-like extensions that partially surrounded the peroxisomes (arrows). However, the complete sequestration of the peroxisomes was not observed. During ethanol adaptation (D), wrapping membranes around a single peroxisome normally seen in wild type cells (see Fig. 2 [9]) were not observed in the gsa11 mutants. N, nucleus; P, peroxisome; V, vacuole.
degrade efficiently AOX during glucose adaptation (Fig. 5A).

However, gsa11 cells expressing GFP/HA-Gsa11(CT) degraded AOX poorly. We next determined whether these truncated forms of Gsa11 became associated with the perivacuolar structures. We have shown that during glucose adaptation, GFP/HA-Gsa11 redistributes from the cytosol to a compartment juxtaposed to the vacuole. The association of GFP/HA-Gsa11 with this compartment was not evident at 1 h of glucose adaptation (Fig. 5B). However, at 3 h GFP/HA-Gsa11 localized to as many as 2–4 structures (Fig. 5C). We then examined the cellular distribution of GFP/HA-Gsa11(NT) and GFP/HA-Gsa11(CT) expressed in gsa11-2 cells. Functionally active GFP/HA-Gsa11(NT) accumulated in the cytosol and perivacuolar structures, while GFP/HA-Gsa11(CT) was largely localized to the vacuole. This suggests that theinteraction between Gsa11 and AOX is crucial for the proper degradation of AOX during glucose adaptation.

**Fig. 3.** Amino acid alignment of Gsa11 and Apg2. The amino acid sequence of Gsa11 from *P. pastoris* was aligned with its structural homologue from *S. cerevisiae*, Apg2 (YNL242w). Amino acid identities (---) and gaps (....) are indicated. The alignment reveals two regions of high homology. The N terminus (1–80 residues) aligns with 55% identity and 72% similarity. There also exists homology at the C terminus with 31% identity and 46% similarity between residues 1045 and 1862 of Gsa11 and residues 888 and 1592 of Apg2.

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HA-Gsa11(NT) was observed in structures juxtaposed to the vacuole (Fig. 5D), but functionally inactive GFP/HA-Gsa11(CT) remained soluble and did not associate with this compartment under these conditions (Fig. 5E). The data suggest that the C terminus of Gsa11 contains information required for its association with this compartment and that this association is likely essential for pexophagy. This region contains a leucine zipper and a putative ATP binding domain, but additional studies are necessary to better define the functional motifs within Gsa11.

Localization of Gsa11 to the Perivacuolar Compartment Requires Gsa10, Gsa12, Gsa14, and PpVps15—Our results suggest that the localization of Gsa11 to a perivacuolar compartment of one or more structures juxtaposed to the vacuole is essential for an intermediate event in the vacuole sequestration of peroxisomes. It is possible that the association of Gsa11 with these structures requires other Gsa proteins. Indeed, the recruitment of Aut7 to the autophagosome membrane in S. cerevisiae has been shown to require many proteins including Apg7, Apg10, Aut1, Aut2, Apg5, and Apg12 (38, 50, 51). In addition, we have shown in S. cerevisiae that Apg2 localizes to one or more perivacuolar structures that contain the integral membrane protein Apg9 and that this localization requires the presence of Apg9 (42). Therefore, in an attempt to define those proteins possibly interacting with Gsa11 and thereby promoting its recruitment to this compartment, we examined the cellular localization of GFP/HA-Gsa11 in six different gsa mutants (Fig. 6). The recruitment of Gsa11 to this compartment was predominantly unaffected in WDK09 (gsa9Δ) and R11 (gsa11Δ) cells. GFP/HA-Gsa9 localized predominantly to the Gsa9 compartment at the vacuole surface in both gsa9Δ and gsa11Δ strains (arrowhead, Fig. 7, A and B). Next, we examined...
the cellular localization of GFP/HA-Gsa9 in gsa11 mutants during glucose adaptation (Fig. 7, C–E). The vacuole formed a cup-like structure that partially engulfed the peroxisomes. GFP/HA-Gsa9 localized to the concave surface of the vacuole at site of peroxisome sequestration. In some cells, GFP/HA-Gsa9 appeared to distribute to the peroxisomes (white arrows, Fig. 7C), although in others it was found to completely surround the peroxisome cluster (white arrows, Fig. 7, D and E). In addition, the GFP/HA-Gsa9 occasionally localized to two structures that were positioned adjacent to the vacuole and at opposite sides of the ring-like structure of GFP/HA-Gsa9 (arrowheads, Fig. 7, D and E). The redistribution of GFP/HA-Gsa9 from the single structure at the vacuole surface to a position around the peroxisomes proceeded independent of vacuole engulfment.

We next examined the distribution of GFP/HA-Gsa9 in R2 (gsa12) and WDY7 (gsa7) mutants, which are blocked at early and late sequestration events, respectively. At 3 h of glucose adaptation, the vacuole in gsa12 mutants was round except for a flattening at the region where it interacted with the peroxisomes (Fig. 8, A and B). The GFP/HA-Gsa9 localized to this flattened region of the vacuole and to the peroxisomes (white arrows, Fig. 8C–E). The GFP/HA-Gsa9 localized to the perivacuolar compartment in the gsa7 and gsa9 mutants but remained cytosolic in the gsa14, gsa12, gsa10, and PpVPS15 mutants.
In *gsa7* mutants, the vacuole was found to almost completely surround the peroxisomes at 3 h of glucose adaptation (Fig. 8, C and D). Meanwhile, GFP/HA-Gsa9 localized around the peroxisome cluster and to the vacuole surface adjacent to the peroxisomes. However, unlike that seen in the *gsa11* mutant, GFP/HA-Gsa9 did not form a ring-like structure around the peroxisome cluster (D and E) (white arrows). The Gsa9 compartment adjacent to the vacuole was now evident at opposite sides of the concave surface of the involuting vacuole (arrowheads).

**DISCUSSION**

*P. pastoris* is capable of adapting to changes in its environment by modifying its protein complement necessary to support growth. For example, when methanol is provided as the sole carbon source, this yeast will synthesize those cytosolic and peroxisomal enzymes required to assimilate this nutrient. However, when the medium is then switched from methanol to glucose or ethanol, the peroxisomal enzymes that are no longer required are rapidly degraded. We have shown previously that during glucose and ethanol adaptation the yeast selectively degrades these peroxisomes within the vacuole by processes called micropexophagy and macropexophagy, respectively (9). In addition, vacuole-mediated protein degradation is enhanced when *P. pastoris* is starved for amino acids and nitrogen. These events proceed by a process analogous to mammalian autophagy, and those proteins required for yeast autophagy appear to be required for mammalian autophagy (52, 53).

We have been examining the molecular events required for glucose-induced micropexophagy in *P. pastoris*. By utilizing the...
REM1 approach to randomly mutagenize the yeast genome and a sensitive direct colony assay to identify mutants, we have been able to sequence a number of GSA genes that are required for micropexophagy (Table I). In this report, we have characterized GSA11, a gene encoding a novel 208-kDa protein. This protein is required for selective micropexophagy and macrophagy, as well as for starvation-induced autophagy, suggesting that these morphologically distinct pathways are molecularly related. Gsa11 is structurally homologous to *S. cerevisiae* Apg2. Apg2 is required for sorting aminopeptidase I from the cytosol to the vacuole and for starvation-induced autophagy (42, 54). In addition, like all *apg* mutants, the diploid null mutant of *apg2* is unable to sporulate arresting at the first meiotic division (55, 56). Planta et al. (57) have shown that the mRNA levels of *APG2* (YNL242W) increase when cell cultures progress from logarithmic to stationary growth in glucose and when starved for amino acids and nitrogen. Gsa11 and Apg2 have structural homologues in many eukaryotes, including *Drosophila* and humans, indicating that a protein with similar functions likely exists in other species.

The selective sequestration of peroxisomes during glucose adaptation occurs at the vacuole surface. Indeed, we have shown previously that Gsa12 and Gsa9 are associated with the vacuole. GFP-Gsa12 is a cytosolic protein that associates with the vacuole membrane, and a single structure at the vacuole surface, which we call the Gsa9 compartment (24). Whereas Gsa9 appears to label the peroxisomes and function at the site of sequestration during micropexophagy, Gsa11 does not associate with the peroxisomes but instead associates with a perivacuolar compartment composed of one or more cytoplasmic structures juxtaposed to the vacuole at the surface opposite where the sequestration of peroxisomes was occurring (Fig. 4). Gsa11 is required for an intermediate event in micropexophagy, and our results suggest that this perivacuolar compartment is probably the functional site of Gsa11. Indeed, the onset of micropexophagy correlates with the association of Gsa11 with this perivacuolar compartment, and the C terminus of Gsa11 is required for its interaction with this compartment as well as for its ability to support micropexophagy. Therefore, the assembly of Gsa11 into this compartment appears to be a prerequisite for an intermediate event in micropexophagy.

The association of Gsa11 with the perivacuolar compartment requires a number of proteins including an integral membrane protein Gsa14, two serine/threonine protein kinases Gsa10 and PpVps15, and Gsa12, a protein containing WD40 protein binding domains. In *S. cerevisiae*, Apg2, the structural homologue of Gsa11, also localizes to one or two structures juxtaposed to the vacuole, and this localization requires Apg9, the homologue of Gsa14. In addition, Apg2 and Apg9 coprecipitate and cofractionate on linear Optiprep gradients (42). Indeed, it is possible that Gsa11 binds to Gsa14 thereby anchoring it to this compartment. Both *P. pastoris* Gsa14 and *S. cerevisiae* Apg9 are integral membrane proteins with 5–7 membrane spanning domains. Although the function of Apg9 is not known, it has been suggested to be a marker for the compartment that recruits and supplies the membrane components to the autophagosome (45). Apg9 is not found on the completed autophagosome, but Aut7, a protein associated with autophagosomes and required for their growth, has been found to localize to this Apg9 compartment (54). In addition, the localization of Gsa11 to the perivacuolar compartment requires Gsa10 and PpVps15. Gsa10 is a serine/threonine protein kinase that is homologous to *S. cerevisiae* Apg1. In *S. cerevisiae*, Apg1 forms a complex
GSA11 Requirement for Pexophagy and Autophagy

with Cvt9 and Apg17, phosphoproteins that are required for selective autophagy and starvation-induced autophagy, respectively (25, 58). Upon nutrient deprivation or inactivation of Tor with rapamycin, alterations in the phosphorylation of Apg13 lead to the association of the Apg1 complex with Apg13. These events have been suggested to be the regulatory switch between selective autophagy of aminopeptidase I and starvation-induced autophagy. Gsa11 localization was unaltered in cells lacking Gsa9 (the *P. pastoris* homologue of Cvt9) suggesting that the association of Gsa11 with this compartment is independent of Gsa10 binding to Gsa9. In *S. cerevisiae*, Vps15 is a serine/threonine protein kinase required for normal phosphoinositide 3-kinase activity of Vps34 (28). The interaction of Vps15 with Vps34 is required for the transport of proteinase A and proteinase B to the vacuole (59), whereas the addition of Vps38 with Apg14 results in phosphoinositide 3-kinase activity specifically required for selective and nonselective autophagy (29). However, Gsa11 does not contain any known phosphoinositide 3-phosphate binding domains suggesting that loss of Gsa11 binding is not a direct effect of the absence of phosphoinositide 3-phosphate.

Our data suggest that the perivacuolar compartment containing Gsa11 differs both structurally and functionally from the Gsa9 compartment. First, the Gsa9 compartment appears as a single complex at the vacuole surface. During glucose-induced micropexophagy, this compartment appears to either divide or multiply by *de novo* synthesis into at least two structures that are positioned at opposite ends of the peroxisome cluster (Fig. 7). The re-positioning of the Gsa9 compartment, which we believe is essential for labeling the peroxisomes for sequestration, does not require Gsa11. The perivacuolar compartment is also positioned near the vacuole, but opposite the site where peroxisome sequestration is ongoing (Fig. 4). In addition, the distribution of Gsa11 to this compartment is unaltered in the absence of Gsa9 (Fig. 6). Finally, the data suggest that the Gsa9 compartment is only required for pexophagy, whereas the perivacuolar compartment is required for both pexophagy and autophagy. Gsa9 is likely required for tethering the vacuole to the peroxisomes during micropexophagy. However, we propose that Gsa11 is required for organizing a perivacuolar compartment that supplies membrane proteins and lipids to the vacuole (or autophagosome) for the sequestration of organelles. Data from *S. cerevisiae* indicate that during starvation-induced autophagy Aut7 is synthesized and possibly assembled into the Apg9 compartment whereby it is then transferred to the growing autophagosome (37, 38, 50, 54, 60). We suggest that the sequestration of peroxisomes by the vacuole requires newly synthesized proteins that transit through this perivacuolar compartment of Gsa11 to the vacuole. Indeed, cycloheximide causes a blockage at the intermediate stage of pexophagy (16). In addition, *gsa11* mutants cannot proceed beyond this intermediate stage. Therefore, we suggest that the association of Gsa11 with the perivacuolar compartment is required for the transfer of proteins and lipids to the vacuole, thereby providing the additional membrane components necessary to allow sequestration to proceed to completion. Further experiments will be needed to identify the molecular components of these compartments before and during pexophagy and to evaluate their functions.

Our most recent study suggests a role for Gsa9 in tethering of the vacuole to the peroxisomes during the engulfment process (24). Upon glucose adaptation, Gsa9 redistributes from its location adjacent to the vacuole to the peroxisomes destined for degradation. We project that Gsa9 may be a protein that allows recognition of the peroxisomes by the vacuole. However, it was unclear if Gsa9 first interacts with the peroxisomes and then the vacuole or with the vacuole and then the peroxisomes. In *gsa11* and *gsa12* cells, we have shown that Gsa9 associates with the peroxisomes despite the inability of the vacuole to engulf peroxisomes (Fig. 8). Therefore, the evidence suggests that Gsa9 interacts with the peroxisomes prior to their interaction with the vacuole surface. Indeed, Veenhuis and co-workers (61) have shown that the degradation of peroxisomal enzymes requires they be packaged within the peroxisome membrane. There exist two coiled-coil proteins, Pep14 and Pep17, present at the surface of peroxisomes that may readily interact with Gsa9, itself a coiled-coil protein. Pep14 and Pep17 are membrane proteins that have been shown to bind to proteins of the docking complex, which is required for the import of proteins into peroxisomes (62, 63). Veenhuis et al. (64) have shown that Pep14 (formally called Per10) is required for the degradation of peroxisomal remnants. Interestingly, nonphosphorylated Pep14p is the primary form present in cells actively synthesizing peroxisomes, whereas the phosphorylated form predominates during peroxisome degradation (12). Pep17 in-

![Fig. 9. The events of glucose-induced micropexophagy.](image-url)
teracts with a number of proteins including Pex14, but its requirement for peroxisome degradation has not been evaluated (63). The data suggest that the association of Gsa9 with the peroxisome is an early event in pexophagy and occurs independent of Gsa11 and Gsa12.

Our data suggest that glucose-induced macrophagy proceeds through five morphologically and genetically defined events which include the following: 1) sequestration signal; 2) early sequestration including peroxisome recognition by Gsa9; 3) intermediate sequestration that requires the assembly of the Gsa11 perivacuolar compartment; 4) late sequestration involving the homotypic fusion of the vacuole membrane; and 5) degradation resulting in hydrolysis of the peroxisome (Fig. 9). We have reported previously that Gsa1 is likely required for degradation resulting in hydrolysis of the peroxisome (Fig. 9).

Pep4 and Prb1 (9).

In the early sequestration stage, vacuole membranes are brought together, and fusion occurs resulting in the incorporation of the peroxisome (Fig. 9). The assembly of the Gsa11 perivacuolar compartment, which is the prerequisite for the continued sequestration of the peroxisome by membranes of unknown origin is inhibited during ethanol-induced macropexophagy. We propose that the cyto-oligand binding but for progression to the intermediate stage. The Gsa11 perivacuolar compartment, which is the assembly of the Gsa11 perivacuolar compartment, which is the prerequisite for the continued sequestration of the peroxisome by membranes of unknown origin is inhibited during ethanol-induced macropexophagy.

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