Cells must regulate both biosynthesis and degradation to ensure proper homeostasis of cellular organelles and proteins. This balance is demonstrated in a unique way in the yeast *Saccharomyces cerevisiae*, which possesses two distinct, yet mechanistically related trafficking routes mediating the delivery of proteins from the cytoplasm to the vacuole: the biosynthetic cytoplasm to vacuole targeting (Cvt) and the degradative autophagy pathways. Several components employed by these two transport routes have been identified, but their mechanistic interactions remain largely unknown. Here we report a novel gene involved in these pathways, which we have named *ATG23*. *Atg23* localizes to the pre-autophagosomal structure but also to other cytosolic punctate compartments. Our characterization of the Atg23 protein indicates that it is required for the Cvt pathway and efficient autophagy but not pexophagy. In the absence of Atg23, cargo molecules such as prApe1 are correctly recruited to a pre-autophagosomal structure that is unable to give rise to Cvt vesicles. We also demonstrate that Atg23 is a peripheral membrane protein that requires the presence of Atg9/Apg9 to be specifically targeted to lipid bilayers. Atg9 transiently interacts with Atg23 suggesting that it participates in the recruitment of this protein.

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used in this study are listed in Table I. For ATG23 gene disruptions, the entire coding region was replaced with either the Escherichia coli kan′ or the S. cerevisiae TRP1 gene using PCR primers containing ~40 bases of identity to the regions flanking the open reading frame. PCR-based integrations of the triple HA tag, the 13xMyc tag, protein A (PA), GFP, and YFP at the 3′ end of ATG23 and ATG9 were used to generate strains expressing fusion proteins under the control of their native promoters. The templates for integration were pFA6a-3HA-TRP1, pFA6a-13Myc-TRP1, pDH3, pFA6a-GFP-TRP1, pFA6a-GFP-KanMX, pFA6a-13Myc-TRP1, pDH3, and pHAB102 (22–24). PCR verification and prApe1 processing were used to confirm the functionality of all genomic fusions.

Plasmids expressing PA (pRS416-CuProtA), CFP-Ape1 (pTS470), YFP-Atg11 (pPS97), CFP-Atg8 (pRS416ECFP-Aut7), and Atg19-CFP (pCVT19CFP(414)) have been used to confirm the functionality of all genomic fusions.

Plasmids expressing PA (pRS416-CuProtA), CFP-Ape1 (pTS470), YFP-Atg11 (pPS97), CFP-Atg8 (pRS416ECFP-Aut7), and Atg19-CFP (pCVT19CFP(414)) have been described elsewhere (19–21, 25, 26).

Cells were grown in rich (YPD: 1% yeast extract, 2% peptone, 2% glucose, amino acids and vitamins as needed). Starvation experiments were performed as described elsewhere (17).

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### RESULTS

**atg23Δ Cells Are Defective in the Cvt Pathway and Impaired in Autophagy**—The resident vacuolar hydrolase aminopeptidase I (Ape1) is synthesized in the cytosol as an inactive precursor protein (prApe1) and then delivered directly across the limiting membrane of the vacuole via the Cvt pathway during growth conditions, where it is processed to the mature, active protein (14, 18, 34, 35). During periods of nitrogen starvation, both Cvt cargo and bulk cytoplasm are transported across the vacuolar membrane by the autophagy pathway (18, 34, 36). We recently performed a systematic screen of the yeast ORF deletion library by Western blot looking for the accumulation of prApe1 in nitrogen-rich conditions to identify novel genes required for the Cvt pathway. One such gene identified was CVT23 (ORF YLR431C). This gene was subsequently renamed ATG23 in accordance with a new, unified genetic nomenclature system for Atg20-related genes in yeast (15). To verify that ATG23 is required for the Cvt pathway, we performed a pulse-chase analysis to determine the kinetics of prApe1 import in atg23Δ cells.

Yeast cells were pulse-labeled with [35S]methionine/cysteine and subjected to a non-radioactive chase. In wild type cells, the 61-kDa prApe1 was processed to the 50-kDa mature form with a half-time of ~30 min (Fig. 1). In contrast, the atg23Δ mutant specifically accumulated prApe1 in rich medium (Fig. 1), demonstrating that atg23Δ cells are indeed defective for the Cvt pathway. To determine whether this block was specific to the Cvt pathway, we examined the delivery of proteins to the vacuole through the carboxypeptidase Y pathway. Many resident vacuolar hydrolases, including Prc1 (carboxypeptidase Y), are delivered to the vacuole through a portion of the secretory pathway (1). We found that the processing of Prc1 was unaffected in atg23Δ cells (data not shown). This result indicates that the atg23Δ mutant did not display a general block in vacuolar protein delivery, implying a specific function for Atg23 in the Cvt pathway.

We wished to determine whether Atg23 had a function in autophagic trafficking to the vacuole, because many of the proteins utilized by the Cvt pathway are also needed for autophagy. Although most currently characterized atg mutants are defective in delivery through both pathways, there is an increasing list of genes required primarily for the Cvt pathway, including VAC8, ATG11/CVT9, ATG20/CVT20, ATG24/CVT13, and the VFT tethering complex (28, 29, 37, 38). As prApe1 can be specifically transported to the vacuole through both the Cvt pathway and autophagy (34), mutants in Cvt-specific genes are
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of autophagosomes. To address these possibilities, we used electron microscopy to examine the ultrastructure of the autophagic bodies that accumulated in \textit{atg23Δ pep4Δ} cells during starvation. Autophagic bodies are single membrane intravacuolar vesicles that result from the fusion of autophagosomes with the vacuole; once delivered to the lumen, they are degraded in a Pep4-dependent manner (42). As seen in Fig. 2D, \textit{pep4Δ atg23Δ} mutants accumulated autophagic bodies of normal size within the vacuolar lumen under starvation conditions. Quantification of the numbers of autophagic bodies, however, revealed approximately a 70% reduction in the number of autophagic bodies accumulating in \textit{atg23Δ} cells compared with wild type (10.5 ± 3.5 for \textit{pep4Δ} cells compared with 3.18 ± 2.1 for \textit{pep4Δ atg23Δ} cells, \textit{n} = 50 vacuoles). This reduction correlated very well with the biochemical data (Fig. 2B) and indicated that \textit{atg23Δ} mutants exhibit decreased efficiency of autophagy rather than a structural defect in autophagosome formation.

Finally, we wished to determine a role, if any, for Atg23 in pexophagy. Excess peroxisomes in \textit{S. cerevisiae} can be delivered to the vacuole for degradation by using machinery that largely overlaps with the Cvt pathway and autophagy; most currently characterized \textit{atg} mutants, including those specific for the Cvt pathway, are defective in this process (27). Cells were grown in oleic acid to induce peroxisome proliferation and then shifted to glucose-containing medium lacking nitrogen to induce maximum degradation of the superfluous organelles. We measured pexophagy in wild type, \textit{atg23Δ}, and \textit{atg9Δ} cells by following the degradation of the peroxisomal thiolase Fox3. As shown in Fig. 3, elimination of Fox3 in \textit{atg23Δ} cells was comparable with wild type, whereas \textit{atg9Δ} cells exhibited no such degradation. This indicates that Atg23 is not required for pexophagy. These results place the \textit{atg23Δ} strain into a unique category of mutants that are defective in the Cvt and autophagy pathways but not in pexophagy.

\textbf{Atg23 Is Required for Cvt Vesicle Formation—}To gain insight into Atg23 function, we decided to identify the step at which Atg23 acts in the Cvt pathway, utilizing the current model of Cvt cargo transport: Cargo selection, sequestration into intact Cvt vesicles, vesicle fusion with the limiting membrane of the vacuole, and maturation of the prApe1 cargo in the vacuolar lumen (reviewed in Ref. 43). Following oligomerization of prApe1 in the cytosol, the Ape1 complex binds to the Atg19/ Cvt19 receptor to form the Cvt complex, which is subsequently recruited to the PAS in an Atg11-dependent step (26, 36). We first assayed whether \textit{atg23Δ} cells were competent to recruit the Cvt complex to lipid bilayers by performing a membrane flotation experiment (Fig. 4A). Following spheroplast lysis, the majority of prApe1 in \textit{atg23Δ} cells was found in the pellet fraction (P13). In contrast, almost all the cytosolic marker Pgk1 was found in the S13 supernatant fraction, indicating efficient lysis of spherooplasts. The prApe1-containing fraction was then subjected to centrifugation on a Ficoll step gradient as described under “Experimental Procedures.” Float fractions were recovered in the absence and presence of detergent and subjected to a Western blot analysis for prApe1. Due to their buoyancy, all lipids, membranes, and associated proteins will float to the top of the gradient; moreover, the addition of detergent disrupts the membranes and abolishes the flotation of membrane-bound proteins. All prApe1 recovered from the \textit{atg23Δ} cells was found in the float fraction in the absence of detergent and not in the presence of detergent. This indicates that prApe1 is membrane- or lipid-associated in the \textit{atg23Δ} strain.

The next step in the Cvt pathway is the sequestration of prApe1 into intact Cvt vesicles. Accordingly, we next analyzed...
were grown to aG23 (SEY6210), and aG23 exhibit intermediate starvation resistance. Wild type (SEY6210), pep4 Cells from the aG23 features in aG13 aG23 partially induced in immunoblot analysis with anti-Ape1 antiserum. B SDS-N medium for 2 h. Protein extracts were prepared and subjected to activity in the other strains normalized relative to wild type. Error bars represent the S.D. from three separate experiments. The ability of aG23 cells to complete the sequestration process through a protease-protection assay (Fig. 4B). If a mutant is blocked at the step of sequestration, Cvt vesicles will not close and prApe1 will accumulate in a form sensitive to exogenous protease following osmotic lysis of the spheroplast plasma membrane. However, if a mutant is competent for sequestration, this hydrolase will be protected from exogenous protease, becoming sensitive only upon membrane disruption by the addition of detergent (17). Spheroplasts were prepared and subjected to osmotic lysis. As before, efficient separation of Pgp1 into the supernatant fraction indicated efficient lysis of spheroplasts. The pelletable cellular fraction from aG23 pep4Δ mutants was subjected to treatment by protease K in the absence or presence of detergent. As shown in Fig. 4B, the accumulated prApe1 in aG23 pep4Δ cells was accessible to the exogenous protease K in the presence or absence of detergent. To verify that this sensitivity is not the result of nonspecific disruption of intracellular membranes, we simultaneously examined the protease sensitivity of endogenous Pho8. Precursor Pho8 is accumulated in the limiting membrane of the vacuole in pep4Δ mutants. The precursor form of Pho8 contains a small cytosolically oriented tail, whereas the major part of the protein, including the C-terminal propeptide, resides within the vacuolar lumen. Treatment with protease K in the absence of detergent allowed the cleavage of only the cytosolic tail (Fig. 4B). Coupled with the fractionation of Pgp1, this result indicated that the experimental conditions selectively disrupted the plasma membrane but maintained the integrity of the vacuole and presumably other intracellular compartments, i.e. protease sensitivity of prApe1 in aG23Δ cells was not due to osmotic lysis of completed Cvt vesicles. Upon the addition of detergent, all intracellular membranes were disrupted, and the entirety of Pho8 became accessible to the exogenous protease K. This can be seen as a second decrease in the molecular mass of Pho8 following SDS-PAGE and Western blot analysis resulting from removal of the C-terminal propeptide. These results indicate a sequestration defect in aG23 pep4Δ mutants. Apg23 Is Not Necessary for Protein Recruitment to the PAS—To continue our analysis of the role of Apg23 in the Cvt and autophagy pathways, we decided to determine whether Apg23 is required for the recruitment of PAS-associated proteins. Although the exact nature and function of the PAS is not yet clear, it appears to play a physiological role in Cvt vesicle and autophagosome formation; many proteins involved in the Cvt pathway and autophagy have been shown to localize at the PAS (19, 20). As shown in Fig. 5, wild type cells showed the typical co-localization of YFP-Apg8, YFP-Apg11, Apg19-CFP, and prApe1 (CFP-Ape1) to the single punc-
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Atg23 functions at the stage of Cvt vesicle formation. A, precursor Ape1 is membrane-associated in atg23Δ cells. The P13 fraction from atg23Δ (KTY14) spheroplasts was collected and loaded on the bottom of a Ficoll step gradient in the presence or absence of the detergent Triton X-100 (Tx). After centrifugation, the float (F) fractions were collected and analyzed by immunoblot using anti-Ape1 antisera as described under “Experimental Procedures.” Lysis conditions were verified by immunoblot analysis using anti-Pgk1. B, precursor Ape1 is protease-sensitive in atg23Δ pep4Δ cells. The 13,000 × g pellet (P13) fraction was collected from atg23Δ pep4Δ (KTY22) lysed spheroplasts, subjected to treatment with proteinase K in the presence or absence of Triton X-100 (Tx), and analyzed by immunoblot using anti-Ape1 antisera as described under “Experimental Procedures.” Lysis conditions were verified by immunoblot analysis using anti-Pgk1 and anti-Pho8 antisera. T, total; S13, 13,000 × g supernatant fraction.

Atg23 is a peripheral membrane protein that localizes to the PAS—In order to gain more information about Atg23, we decided to examine the biosynthesis of the protein. Many of the components involved in the Cvt and autophagy pathways are peripheral membrane proteins that specifically associate with the PAS; as the amino acid sequence of Atg23 yielded no obvious membrane-spanning domains, we hypothesized that this protein may also have similar characteristics. To monitor the localization of Atg23, we generated a functional C-terminal Myc fusion at the chromosomal ATG23 locus. Immunoblot analysis with anti-Myc antiserum detected a protein with the predicted molecular mass. The Myc tag did not interfere with Atg23 function because the strain with the integrated tag displayed normal maturation of prApe1 (data not shown). The strain expressing Atg23-Myc was subjected to subcellular fractionation into soluble and pelletable fractions. As shown in Fig. 6A, the majority of cellular Atg23-Myc was found in the soluble fraction (S100); however, a significant portion of the protein was also found in both the P13 and P100 fractions. In contrast, cytosolic Pgk1 was completely absent from the P13 pellet, again indicating efficient lysis of spheroplasts and separation of the pelletable and soluble fractions. This distribution of Atg23 is similar to other Atg proteins that are peripherally associated with membranes such as Atg2, Atg11, and Atg18 (28, 33, 44).

Atg23 is not required for the organization of proteins at the PAS—Wild type and atg23Δ (KTY14) cells were co-transformed with plasmids expressing YFP-Atg11 or YFP-Atg8 and Atg19-CFP or CFP-Ape1, grown in selective SMD medium to mid-log phase and visualized by fluorescence microscopy. DIC, differential interference contrast.

Atg9 is essential to recruit Atg23 to membranes and both proteins localize to multiple distinct punctate structures in the cytoplasm—To follow the localization of Atg23 in vivo, we visualized the localization of this protein in living cells by fluorescence microscopy (Fig. 7A). The gene encoding YFP was integrated in-frame at the 3′ end of ATG23 at the chromosomal locus, and the resulting Atg23-YFP fusion protein was functional based on the ability of this strain to import prApe1 (data not shown). Cells expressing both Atg23-YFP and the PAS marker Atg19-CFP were grown to early log phase and imaged with a fluorescent microscope. Atg23-YFP localized to multiple, distinct structures dispersed throughout the cytoplasm and also displayed some diffuse cytoplasmic staining. This distribution was identical under both growth and starvation conditions (data not shown). Co-localization with Atg19-CFP indicated that one of those punctate structures was the PAS (Fig. 7A), consistent with the results from the gradient analysis. The
localization pattern of Atg23 was unusual in that it resembled the pattern displayed by the transmembrane protein Atg9 (21), a pattern distinct from other Atg proteins.

Because of this similarity, we decided to examine if Atg23 and Atg9 associated with each other. Accordingly, we utilized the strain expressing the Atg9-PA and Atg23-HA fusions. These cells were converted to spheroplasts, lysed, and the PA chimera isolated using IgG-Sepharose beads. Bound complexes were released from the Sepharose by eluting with a low pH buffer, and the presence of Atg9-PA, Atg23-HA, and Pgk1 was tested by immunoblotting. As shown in Fig. 7B, Atg23 was selectively affinity-isolated by the Atg9-PA fusion, demonstrating an interaction between these two proteins. The presence of Atg23-HA in the eluates was not caused by nonspecific binding to the IgG-Sepharose or PA because the abundant cytosolic protein Pgk1 was not detected in the same samples, and PA alone did not pull down the Atg23-HA fusion.

We then examined if the association between Atg23 and Atg9 was important for their proper localization. Atg9-YFP was expressed in atg23Δ cells, and conversely, Atg23-GFP was examined in the atg9Δ strain. As shown in Fig. 7C, the absence of Atg23 did not interfere with normal Atg9-YFP distribution. In contrast, the punctate localization of Atg23-GFP was completely lost in the atg9Δ mutant and was instead found solely in a diffuse cytosolic pool. Reintroducing plasmid-based Atg9 restored the wild type localization of Atg23-GFP, indicating that its altered cellular distribution was due to the ATG9 deletion (data not shown). We concluded that Atg9 is essential to recruit Atg23 to membranes.

DISCUSSION

Atg23 Is Required for the Cvt Pathway and Efficient Autophagy but Not Pexophagy—The Cvt pathway and autophagy are two vacuolar trafficking pathways that serve drastically different purposes. The Cvt pathway is a biosynthetic trafficking pathway for a distinct subset of resident vacuolar hydrolases, whereas autophagy is a nonspecific degradative mechanism to allow cell survival during starvation. However, the two pathways are morphologically very similar and share a number of identical molecular components. To identify currently unknown genes required for these two pathways, we undertook a screen to identify deletion mutants defective for prApe1 import during growth conditions, and we identified a strain that was disrupted for the ATG23 gene. This gene codes for a 453-amino acid protein that contains three putative coiled-coil domains with clear homologues in the Saccharomyces fungus family but not in other eukaryotic organisms.

The characterization of the atg23Δ strain revealed a phenotype distinct from all other reported Atg deletion mutants. We demonstrated that Atg23 is used by both the Cvt pathway and autophagy; however, unlike most previously characterized ATG genes, it is not absolutely required for autophagy (Fig. 2, A and B). Mutants lacking Atg23 can form morphologically normal autophagosomes and deliver them to the vacuole, but at too slow a rate to sustain viability during extended periods of starvation (Fig. 2, C and D). Excess peroxisomes in S. cerevisiae can be sequestered into double membrane-bound structures and delivered to the vacuole in a manner similar to autophagy. This process is in unaffected in the atg23Δ mutant (Fig. 3). Thus, atg23 falls into a relatively unique category of mutants that is not defective in pexophagy. This group includes atg19Δ, for example; ATG19 encodes the receptor for prApe1 import and also displays normal autophagy (36).

The gene products specific for the Cvt pathway can be divided in two classes. The first class, consisting of factors required to target cargo molecules to the PAS (26), are proteins with homologues only in other fungi (43). The second class includes components with a role in vesicular traffic; these proteins may be involved in mediating membrane de-
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Atg23 Recruitment to Membranes Depends on Atg9—Many Atg proteins localize to a single punctate structure (19, 20). The analysis of a fluorescent Atg23 fusion revealed that this protein is concentrated to several punctate structures dispersed in the cytosol (Fig. 7A). Among all the Atg components, Atg9 is the only factor that displays this unique localization pattern (21). Only one of these structures corresponds to the PAS, indicating that these proteins are divided in two qualitatively different populations. Surprisingly, membrane separation by sucrose step gradients showed that Atg23 and Atg9 exclusively co-fractionated in a single peak (Fig. 6B). It has been demonstrated previously that those Atg9-containing fractions also include several other Atg components, confirming our fluorescence microscopy data (19). We attempted to separate the two Atg9 and Atg23 populations by differential subcellular centrifugation and OptiPrep™ linear and sucrose step gradient fractions, but we were unsuccessful (data not shown). This suggests that the punctate structures have a very similar, if not identical, density and size.

Because of the similarity between Atg23 and Atg9 localization, we investigated if those two molecules were associated. Affinity isolation experiments demonstrated that Atg23 can indeed bind Atg9 (Fig. 7B). Cells contain both a soluble and a membrane-bound pool of Atg23 suggesting that it is a peripheral membrane protein (Fig. 6A). Interestingly, in the absence of Atg9, Atg23 lost its punctate localization, becoming completely dispersed (Fig. 7C). The simplest hypothesis would be that Atg23 is associated with membranes via its binding to the integral membrane protein Atg9. However, three separate results indicate that Atg9 catalyzes Atg23 recruitment to membranes in a process temporally and locally restricted to the PAS. First, Atg9 is accumulated at the PAS in the atg2Δ, atg18Δ, and atg14Δ mutants, whereas Atg23 has normal distribution. That result indicates that the Atg23 pool not localizing to the PAS does not require the physical presence of Atg9 to remain membrane-associated. Second, Atg23 was affinity-isolated by Atg9 in atg1Δ cells when both proteins were restricted to the PAS. Third, Atg23 is completely cytosolic in the atg1Δ atg9Δ double deletion mutant showing that Atg9 mediates the targeting of Atg23 to the PAS (data not shown). The conclusion that Atg23 and Atg9 do not form a functional unit is also supported by the difference in phenotypes between the corresponding deletion mutants. For example, the Cvt pathway, autophagy, and pexophagy are completely blocked in atg9Δ cells (21) (Fig. 2A and Fig. 3). In addition, Atg8 is not concentrated at the PAS in the atg9Δ mutant, whereas it normally localizes to this structure in atg23Δ cells (20) (Fig. 5). It remains to be established if the pools of Atg9 and Atg23 not localizing to the PAS are in the same location. Our co-localization attempts with YFP and CFP fusions of these two proteins failed because of the weak signal.2

Future work will try to identify other Atg23-binding partners in order to unveil the role played by this protein in the Cvt pathway and autophagy. In addition, the study of the relationship between the Atg23 pools at the PAS and at the other punctate structures will provide insights about both the origin and the delivery mechanism of the membranes composing the double-membrane vesicles that are the hallmark of the Cvt and autophagy pathways.

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