Vacuolar aminopeptidase 1 is transported to the vacuole by cytoplasmic double-membrane vesicles, the nonclassic Cvt pathway. The cytosolic protein dodecamerizes and is enclosed in a double-membrane vesicle, which is transported to and fuses with the vacuole releasing a single-membrane autophagic body into the vacuolar lumen. This is degraded and the precursor sequence of aminopeptidase 1 is removed. This pathway resembles autophagy, and most proteins identified to function in the Cvt pathway are also required for autophagy and vice versa. The cytosolic precursor protein and the matured vacuolar protein form a homododecamer complex, and only this complex has enzymatic activity. We developed a new genetic screen to isolate mutants in the biogenesis of vacuolar aminopeptidase 1 based on its enzymatic activity. The sensitivity of this assay made it possible for us to search for mutants under conditions where autophagy is down-regulated, and we describe two new mutants defective in the biogenesis pathway of vacuolar aminopeptidase 1. Mutants are defective in dodecamerization of pApe1p and in Cvt vesicle formation. Complex assembly and transport vesicle formation appear to be linked processes. This mechanism can control the potentially harmful cytoplasmic proteolytic activity and could be the driving force for this nonclassic mechanism of vacuolar enzyme transport.

Most vacuolar hydrolases are transported to the vacuole through the endoplasmic reticulum and the Golgi apparatus. There are two vacuolar enzymes known, which do not follow this pathway and are transported from the cytoplasm to the vacuole independent of the secretory pathway: aminopeptidase 1 (Ape1p) and α-mannosidase (1, 2). The primary structure of N-terminal presequence of the Ape1p precursor protein (pApe1p) indicated that the protein might not enter the endoplasmic reticulum (3). Biochemical as well as genetic analysis of pApe1p transport by Klionsky and colleagues (2) revealed that pApe1p is transported to the vacuole independently of the Golgi or the secretory plasma membrane route. A genetic screen based on the detection of accumulated Ape1p precursor peptide by Western blot lead to the isolation of Cvt mutants, defective in cytoplasm to vacuole transport of pApe1p (4). These mutants accumulate pApe1p in small double-membrane vesicles, Cvt vesicles, similar to autophagocytic vesicles. Other genetic screens for cytoplasm to vacuole protein transport are designed to study the degradative protein transport pathways essential for yeast growth under nutrient starvation conditions. Mutants unable to survive nutrient starvation were analyzed for defects in autophagocytosis and accumulation of double-membrane vesicles (apg mutants) (5). A cytoplasmic protein degraded by this pathway is fatty acid synthetase, and transport mutants for this protein were isolated (aut mutants) (6). The cytosolic fructose-1,6-bisphosphatase can be degraded in vacuoles, and mutants of this pathway (oid) were also isolated (7). The vacuolar biogenesis-defective Cvt mutants and the mutants of the autophagic pathways overlap genetically, demonstrating that these pathways share components (8, 9).

The pApe1p monomer assembles with a half-time of 2 min into a homododecameric complex in the cytoplasm, but the half-time for vacuolar delivery is 45 min (10). pApe1p has been detected in cytoplasmic double-membrane vesicles by immunoelectron microscopy, and pApe1p is seen in the vacuole of protease-deficient cells within single-membrane vesicles, indicating that the cytosolic pApe1p dodecamer is enwrapped by the membrane of an autophagosome, which is transported to and fuses with the vacuolar membrane (11–13). The 61-kDa cytoplasmic pApe1p is proteolytically matured to a 55-kDa intermediate form and to the 50-kDa vacuolar mApe1p form by the sequential action of the two vacuolar endopeptidases PrA and PrB (14). The 45-amino acid precursor sequence forms a helix-turn-helix structure. Mutations that disturb formation of the first helix also abolish pApe1p transport (15, 16). Ape1p is only found as homododecameric complexes, and the dodecameric state is required for its enzymatic activity (17). The HSC70 proteins Ssa1p and Ssa2p are required for pApe1p transport, however, they do not control dodecamerization of pApe1p but are required for vacuolar transport of pApe1p by Cvt vesicles (18).

We developed a new genetic screen for analyzing the biogenesis pathway of aminopeptidase 1 based on the enzymatic activity of the dodecameric complex. We isolated two new mutants deficient in aminopeptidase 1 transport. Both display phenotypes, which have not been described for Cvt or Apg and Aut mutants. The mutants were termed uia for vacuolar import and autophagocytosis. Mutants are defective in dodecamer for-
mation and pApe1p accumulates in Cvt vesicles, indicating that complex assembly and transport vesicle formation are linked processes. Dodecameric pApe1p is already proteolytically active, and, therefore, packaging of pApe1p into Cvt vesicles is a pathway by which the cell can control the potentially harmful proteolytic activity by controlled assembly and compartmentalization.

MATERIALS AND METHODS

**Strains, Media, and Plasmids—**SEY6211: MATa, ura3-52, his3Δ1, leu2-3,112, trp1-901, Ade2-Δhis, ade2 (S. Emr); J17: MATa, ura3-52, his3Δ1, leu2-3,112, trp1-901, lacY1, lacZΔM14, Δhis (D. H. Wolfe). These were mated and spores were isolated to generate the strains PSY of a and α mating types deficient in all four lap genes. These were transformed with the APE1 gene to generate the strains C6C MATa PSY and C6A MATa.

Media were used as follows. YPD: 1% bacto-yeast extract, 2% bacto-peptone, 2% glucose or 3% ethanol for YPE. MV-D: 0.67% yeast nitrogen base w/o amino acids, 2% glucose. MV-N: 0.17% yeast nitrogen base w/o amino acids, 2% glucose. All amino acids were added to the MV media except the auxotrophic markers required. 2% agar was added for plates. Media ingredients were purchased from Life Technologies, Inc. Cell growth was determined by determining the optical density at 600 nm and in triplicate.

The APE1 genomic locus, including the promoter region, was isolated from a genomic DNA library (gift of H. D. Schmidt) by hybridization of the library with a polymerase chain reaction-amplified APE1 fragment. The locus was subcloned as a 5-kb SalI fragment into the pRS centromere plasmids 313, 314, and 316. Mutations of the PEP4 gene were done with the pTIS1 construct (19).

**Mutagenesis and Colony Screening—**EMS mutagenesis was generated with stationary cultures of C6C in YPD as described. Cells were resuspended in 0.1 M NaP, pH 7.4, 100 μM EMS (Merck) was added to 3 ml cells, and suspension was incubated over 30 min at 30 °C. Cells were washed three times with water, plated onto YPD plates, and incubated for 3 days at 30 °C. Replicates were made on MV-D-Trp and incubated 4–7 days at 30 °C, and colonies were transferred onto filters. These were placed with the colony side up on YPD plates and incubated over night at 30 °C. For colony activity assays colonies were lysed in chloroform, air dried, and covered with 0.7% agarose, 50 mM Tris/Cl, pH 7.5, 3 mM EDTA, 10 mM leucine-β-naphthylamide. Activity was tested at 365 nm after incubation over 10 and 30 min at 25 °C. Fluorescence was recorded by a CCD chemiluminescence camera system (Raytest, 365 nm) after incubation over 10 and 30 min at 25 °C. Fluorescence was recorded by a CCD chemiluminescence camera system (Raytest, 365 nm).

**Protease Protection and Complex Assembly—**Spheroplasts were harvested, resuspended with DEAE-dextran as described above, but no protease inhibitors were included. The lysed material was centrifuged at 5000 × g over 5 min and lysis was analyzed by Western blot analysis of pellet and supernatant for CPY (vacuolar) and hexokinase. The lysed material was incubated at 4 °C over 30 min without adding protease, in the presence of trypsin or proteinase K (each 50 μg/ml) and in the presence of either proteinase and 0.2% Triton X-100. Incubation was terminated by adding 1 volume of 20% trichloroacetic acid/80% acetone. Processing of pApe1p was analyzed after SDS-PAGE by Western blotting.

**Growth**

Growth gradients were done as described previously (10). A step-gradient was formed by equal volumes of 50, 40, 30, and 20% glycerol in 20 mM potassium PIPES, pH 6.8. 1.5 mg/400 μl of crude cell extract proteins were loaded and centrifuged in a TLS-55 rotor at 55,000 rpm over 4 h at 15 °C in a Beckman table-top ultracentrifuge. Gradient was collected in 10 fractions, and proteins were trichloroacetic acid-precipitated and separated on a 10% SDS-PAGE and analyzed by Western blot analysis. Ovalbumin (45 kDa), bovine serum albumin (65 kDa), and thyroglobulin (669 kDa) were used to follow separation on the gradient.

**Vacuole Staining—**Vacuoles of cells were stained with mid-log cultures in YPD. Cells were harvested and resuspended in 50 mM sodium citrate, pH 4, 1 μl of 10 mM CL2CFDA (Molecular Probes) in Me2SO was added, and the cells were incubated at 30 °C over 10 min. Cells were harvested and resuspended in 0.1 M NaP, pH 7.5, 2% glucose and visualized using a Zeiss Axiovert 100 microscope equipped with a Plan-Neofluar 63x/1.25 and differential interference contrast.

**RESULTS**

**Isolation of Aminopeptidase 1 Transport Mutants—**Ape1p is synthesized as a cytosolic precursor protein, pApe1p, which assembles into a homododecamer and is proteolytically matured after it reaches the vacuolar lumen. Only this dodecameric complex is enzymatically active (17). We designed a screen for mutants deficient in the biogenesis pathway of Ape1p based on the enzymatic activity. The sensitivity of the detection method allowed to screen under conditions where autophagy is down-regulated. Cells were grown on filter paper soaked with rich medium (YPD), lysed with chloroform, and filters were covered with buffered 0.7% agarose containing the Ape1p substrate leucine-β-naphthylamide. Enzymatic activity was detected by the β-naphthylamide fluorescence. Yeast contained four leucineaminopeptidase activities (APE1–APE4). To assay Ape1p activity, a plasmid carrying the APE1 gene under the control of its endogenous promoter was transformed in a yeast strain lacking these four leucineaminopeptidase activities (21). When this strain was transformed with one or two single-copy APE1 plasmids, the difference in the copy numbers was clearly detectable by the fluorescence in the overlay assay for enzymatic activity. The overlay assay allowed us to visually detect differences of 20% of wt Ape1p activity (Fig. 1D). This observation was confirmed by determination of specific Ape1p enzymatic activities in cell extracts. Isogenic strains of opposite mating type were deficient in the four enzymes. Enzymatic activities were generated by mating II-17 with SEY6211. Spores were tested for leucineaminopeptidase activities using the overlay assay and recorded with a CCD chemiluminescence camera system.

Leucineaminopeptidase-deficient strains do not show any phenotype related to vacuolar function as for example growth with nitrogen limitation and sporulation.
The strain C6C carrying the APE1 gene on a single-copy plasmid with tryptophan as auxotrophic marker was mutagenized by EMS treatment (Fig. 1A). Cells were grown on rich media (YPD) plates for 2–4 days at 30 °C. Replicas were made on synthetic media (MV-D) containing all supplements except tryptophan to select for the ectopic APE1 gene (Table I). We did not isolate multiple alleles of the mutated genes, indicating that the screen is not saturating and that mutations in this pathway are synergistically lethal with mutations in other genes. We obtained 14 mutants, which were named via mutants for vacuolar import and autophagocytosis mutants. Three rounds of tetrad analysis of the mutants were performed using the isogenic wt strains to isolate spores whose phenotype was caused by a single mutation. We describe the analysis of two complementation groups, via10 and via14 (Fig. 1B). Diploids of the via10 and via14 have 80% of aminopeptidase 1 activity. This could be due to a gene dosage effect and indicates related functions of the gene products.

via10 and via14 Mutants Accumulate pApe1p—Mutants were isolated based on the absence of Ape1p enzymatic activity. After tetrad analysis, via14 cells did not show enzymatic activity, whereas via10 cells had 20% of the wt activity. Crude cell extracts were prepared from the strains in the presence of protease inhibitors to analyze pApe1p processing. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes, and Ape1p was detected using a rabbit anti-Ape1p antiserum (Fig. 2). Cells were grown at 30 °C in nutrient-rich medium (YPD) where autophagy is not active during vegetative growth. wt yeast show 10–30% pApe1p and 70–90% mApe1p under logarithmic growth conditions between $A_{600} = 0.8–1.2$ (YPD), whereas only 2% pApe1p can be detected in stationary cultures with $A_{600} > 2$. via10 accumulates 60% of Ape1p as pApe1p, whereas via14 accumulates 90% as pApe1p under logarithmic growth conditions in nutrient rich medium (YPD). Under stationary growth conditions in YPD medium, pApe1p levels are reduced in via10 and in via14 and mApe1p is detected even in via14, indicating that up-regulation of autophagy by glucose starvation in via10 and via14 cells restores pApe1p transport. Expression of pApe1p is induced by the starvation conditions, as is the case for all vacuolar enzymes and proteins related to vacuolar function (26), but vacuolar transport is not comparably induced, so that pApe1p accumulates. The ratio of pApe1p to mApe1p does change, and the majority of Ape1p is present as mApe1p in stationary cultures. Cells were grown in nitrogen starvation medium (MV-N) where autophagy is up-regulated, and we tested for pApe1p (Fig. 2). In nitrogen starvation medium pApe1p transport was enhanced in both via mutants, leading to mApe1p levels in via10 comparable to wild-type and in stationary via14 cells comparable to mApe1p in logarithmically growing wt cells. Note that we loaded 10 µg of wt cell extract and 20 µg of mutant cell extracts on the gels used for Fig. 2, as can be seen on the CPY loading control. This allowed the quantitative comparison of the Ape1p signal intensities despite the differences in expression levels between wt and via mutant cells.

Figs. 2 and 4 demonstrate the reduced expression levels of Ape1p in the via mutants. This could be caused by enhanced proteolytic turnover within the vacuole due to misfolding or disturbed complex assembly. Mutation of the vacuolar endopeptidase PrA (PEP4) prevents degradation of the vacuolar autophagic bodies leading to the accumulation of pApe1p within the vacuole (22). We mutated the PrA gene PEP4 and tested for steady-state concentrations of Ape1p in YPD. We found twice as much Ape1p in wt as well as mutant extracts, so the difference between wt and mutant cells remained (not shown). Therefore, vacuolar turnover did not contribute to the lowered Ape1p levels in the mutant cells and degradation by cytoplasmic proteolytic systems should account for the reduced expression levels (Fig. 3). We also tested for APE1 mRNA and

Fig. 1. A, flow chart for the isolation of via mutants (see text for details). B, Ape1p “overlay” activity assay used for the isolation of Ape1p transport mutants. The image was recorded at 365 nm using a CCD chemiluminescence camera. Strains with Ape1p enzymatic activity emit fluorescent light, and colony streaks become visible. Strains shown: OM is the original via14 mutant isolated from the screen. VIA14b spore is positive and VIA14a negative for Ape1p activity. C6C is the wt strain. Diploids for complementation analysis are the via14a/C6C wt cross and the cross of via14a/via10a, a via10 spore with reduced Ape1p enzymatic activity.
which is in agreement with previous results (13). The same majority of pApe1p was found in the pellet fraction of wt cells, supernatant fractions for the vacuolar carboxypeptidase Y of the lysate at 5000 g dextran. Lysis of spheroplasts was controlled by centrifugation (YPD) were converted into spheroplasts and lysed with DEAE-Dextran. Analysis of Ape1p transport. Steady-state levels of pApe1p and mApe1p are detected by Western blot analysis of protein extracts prepared from logarithmically (L) and stationary (S) cultures grown in rich media (YPD) and nitrogen starvation medium (MV-N). Mutant strains express lower amounts of Ape1p. To allow comparison of Ape1p signal intensities, different amounts of cell extract proteins were loaded per lane: 10 μg of wt and 20 μg of mutant cell protein extracts. The vacuolar CPY served as internal control. Protein antibody complexes were detected by chemiluminescence and x-ray film exposure. could not detect differences between mutants and wt cells (Ref. 28 and data not shown). In addition, expression of Ape1p is induced in the mutant cells by starvation conditions as the vacuolar CPY, indicating normal regulation of protein expression (Fig. 2). Induction of pApe1p transport by autophagy in nitrogen starvation medium protects pApe1p from degradation in the cytoplasm (Fig. 2).

pApe1p Accumulates in Cvt Vesicles—We analyzed whether the pApe1p in the via mutants accumulates in vesicles by testing the processing of the precursor protein through added trypsin or proteinase K in the presence or absence of detergent (Fig. 3A). Logarithmically growing cells from rich medium (YPD) were converted into spheroplasts and lysed with DEAE-Dextran. Lysis of spheroplasts was controlled by centrifugation of the lysate at 5000 × g and Western blot analysis of pellet and supernatant fractions for the vacuolar carboxypeptidase Y (CPY) and cytoplasmic hexokinase (HK). mApe1p and the majority of pApe1p was found in the pellet fraction of wt cells, which is in agreement with previous results (13). The same distribution of Ape1p proteins was found in the via10 mutant. In the via14 mutant the entire pApe1p was found in the supernatant after cell lysis (Fig. 3A).

The lysed cells were incubated for 30 min at 4 °C without proteinase added, with added trypsin or proteinase K and with trypsin or proteinase K in the presence of Triton X-100. In wt cells and in the two via mutants, the same amount of pApe1p was protease-protected in the absence of detergent (Fig. 3A). Trypsin is not able to process pApe1p from wt cells in the presence of detergent. pApe1p accumulating in via10 and via14 cells is readily processed by trypsin. pApe1p in wt cells was processed by proteinase K in the presence of detergent. This indicated that pApe1p accumulates in a non-native conformation in via10 and via14 mutants.

We also tested whether endogenous proteases are able to process pApe1p under the in vitro conditions of the protease protection assay by just adding detergent and no additional protease. Strains with a deleted PrA gene (Δpep4) served as control. pApe1p processing of the wt strain was not detectable (Fig. 3B). pApe1p accumulated in via10 and via14 was processed; however, this was not dependent on PrA activity, as expected from the Ape1p levels detected in nitrogen starvation medium and in Δpep4 stains. This demonstrates that the degradation of Ape1p in the via mutants is caused by nonvacuolar proteases.

pApe1p in via14 did not cofractionate with the vacuolar CPY and, therefore, accumulates in prevacuolar vesicles. To analyze whether pApe1p in the via10 mutant accumulates in prevacuolar vesicles, organelles were fractionated on a ficoll gradient and pApe1p and mApe1p fractionation was analyzed as previously described. In wt cells vacuoles and mApe1p are found at the 0–4% Ficoll interphase, whereas Cvt vesicles and pApe1p are found in the pellet fraction (13). Ape1p of via10 migrated into the pellet of the ficoll gradient while mApe1p migrated to the 0–4% Ficoll interphase as in wt cells (Fig. 4A).

In the via14 mutant pApe1p accumulates in prevacuolar vesicles as in wt cells and via10, but they have a lower density than wt Cvt vesicles and are found in a 5000 × g supernatant fraction (Fig. 3A). We tried to pellet the via14 vesicles at 100,000 × g, but pApe1p was not protease-protected anymore after the centrifugation, indicating that these vesicles are fragile (not shown). Therefore, we spun the vesicles onto a 10% Ficoll cushion at 100,000 × g, 4 °C, and 30 min (Fig. 4B). 50% of pApe1p was found at the 10% Ficoll fraction, 30% were in the supernatant, and no pApe1p was found in the pellet. pApe1p in the supernatant was non-protease-protected, but pApe1p in the Ficoll fraction was vesicular, although 50% of the fraction was degraded in the absence of detergent (Fig. 4C). via14 vesicles appear to be extremely fragile (compare Figs. 3A and 4B), indicating that they lack essential components or that their assembly is disturbed.

Deficient Cvt Vesicle Formation—Enhanced cytoplasmic degradation of pApe1p and vesicular accumulation of pApe1p

| Table I Ape1p Oligomerization and Transport
| Statistics of via mutant selection
| | colonies on YPD | colonies on MV | clones without activity | clones with CPY-activity | clones without activity | clones with red activity | after retransformation 14 mutants |
| --- | --- | --- | --- | --- | --- | --- | --- |
| WT | 135000 | 80000 | 850 | 150 | 93 | 57 | ? |
| via10 | 135000 | 80000 | 850 | 150 | 93 | 57 | ? |
| via14 | 135000 | 80000 | 850 | 150 | 93 | 57 | ? |
| L | 100 % | 60 % | 0.6 % | 0.1 % | 0.07 % | 0.04 % | ? |
pApe1p Oligomerization and Transport

The vacuolar endopeptidase proteinase A or PrA is required for the maturation of vacuolar hydrolases and Cvt vesicles, and it is not clear why such an additional pathway exists. The potentially harmful proteolytic activities of vacuolar enzymes are controlled by their pro sequences and compartmentalization. Pro sequences inhibit their enzymatic activities, and they are only processed upon reaching low pH compartments as the endosome and vacuole. In contrast the proteolytic activity of the cytoplasmic proteasome is controlled by chaperones and by selection and modification of the proteins to be degraded by ubiquitination. Initial studies on the four leucineaminopeptidases in Saccharomyces cerevisiae indicated that pApe1p (or LAP IV) activity is also regulated by processing through the vacuolar endopeptidase proteinase A (21). Our data of the pApe1p transport mutants indicate that Cvt vesicle formation controls enzymatic activity of the peptidase through the regulated dodecamerization and compartmentalization. Dodecamerization is required for their enzymatic activity, and it appears to be linked to Cvt vesicle formation. However, this would only be a control mechanism, if the dodecameric precursor pApe1p already has enzymatic activity.

The vacuolar endopeptidase proteinase A or PrA is required for the maturation of vacuolar hydrolases and Cvt vesicles, and autophagosomes are not degraded and accumulate as autophagosomes on a Ficoll gradient. To test whether this transport also restores enzymatic activity and thus dodecamerization of Ape1p, cell lines were grown on YPD and nitrogen starvation medium to logarithmic and stationary phases, and colony overlay assays for Ape1p enzymatic activity were performed (Fig. 7). Although Ape1p enzymatic activity in wt cells increased with incubation time in both media and was higher in nitrogen starvation medium compared with rich medium, via10 did not show an increase of activity and via14 did not have Ape1p enzymatic activity under any condition (Fig. 7).

Cvt Pathway as a Mechanism to Control pApe1p Proteolytic Activity—The Cvt pathway of aminopeptidase 1 is a nonclassic pathway for the transport of vacuolar enzymes and vacuolar biogenesis, and it is not clear why such an additional pathway exists. The potentially harmful proteolytic activities of vacuolar enzymes are controlled by their pro sequences and compartmentalization. Pro sequences inhibit their enzymatic activities, and they are only processed upon reaching low pH compartments as the endosome and vacuole. In contrast the proteolytic activity of the cytoplasmic proteasome is controlled by chaperones and by selection and modification of the proteins to be degraded by ubiquitination. Initial studies on the four leucineaminopeptidases in Saccharomyces cerevisiae indicated that pApe1p (or LAP IV) activity is also regulated by processing through the vacuolar endopeptidase proteinase A (21). Our data of the pApe1p transport mutants indicate that Cvt vesicle formation controls enzymatic activity of the peptidase through the regulated dodecamerization and compartmentalization. Dodecamerization is required for their enzymatic activity, and it appears to be linked to Cvt vesicle formation. However, this would only be a control mechanism, if the dodecameric precursor pApe1p already has enzymatic activity.

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Cvt Pathway as a Mechanism to Control pApe1p Proteolytic Activity—The Cvt pathway of aminopeptidase 1 is a nonclassic pathway for the transport of vacuolar enzymes and vacuolar biogenesis, and it is not clear why such an additional pathway exists. The potentially harmful proteolytic activities of vacuolar enzymes are controlled by their pro sequences and compartmentalization. Pro sequences inhibit their enzymatic activities, and they are only processed upon reaching low pH compartments as the endosome and vacuole. In contrast the proteolytic activity of the cytoplasmic proteasome is controlled by chaperones and by selection and modification of the proteins to be degraded by ubiquitination. Initial studies on the four leucineaminopeptidases in Saccharomyces cerevisiae indicated that pApe1p (or LAP IV) activity is also regulated by processing through the vacuolar endopeptidase proteinase A (21). Our data of the pApe1p transport mutants indicate that Cvt vesicle formation controls enzymatic activity of the peptidase through the regulated dodecamerization and compartmentalization. Dodecamerization is required for their enzymatic activity, and it appears to be linked to Cvt vesicle formation. However, this would only be a control mechanism, if the dodecameric precursor pApe1p already has enzymatic activity.
agic bodies within vacuoles deficient in PrA. This leads to the accumulation of pApe1p in the cells. We tested the \textit{D}pep4 mutants of wt and \textit{via} mutants for aminopeptidase 1 enzymatic activity (see Fig. 3B). wt cells deficient for PrA had full aminopeptidase 1 enzymatic activity, whereas \textit{via10} and \textit{via14} were still deficient for aminopeptidase 1 activity, demonstrating that the pro sequence of aminopeptidase 1 does not control the proteolytic activity of the enzyme (Fig. 8).

Vacuolar Function and Autophagy in \textit{via} Mutants—Accumulation of cytoplasmic pApe1p Cvt transport vesicles could also be caused by impaired vacuolar functions, and we therefore tested the mutants for functional vacuoles. We first analyzed vacuolar morphology and acidification by phase contrast microscopy and staining of the vacuoles with the pH-sensitive fluorescent dye DC\textsubscript{1}LFDA (Fig. 9A). This did not reveal differences in vacuolar morphology between the strains irrespective of the growth stage or media (Fig. 9A shows cells from YPD at \(A_{600} = 1\)), indicating that biogenesis of the target organelle for pApe1p transport is not effected in the mutants. Intact cell and vacuolar morphology of the \textit{via10} and \textit{via14} mutants indicates that osmotic balance is not effected in the mutants. To verify this effect, cell lines were grown in the presence of 0.5, 1, and 1.5 M NaCl in YPD medium. wt and both \textit{via} strains displayed the same sensitivity to osmotic stress and grew slower with increasing salt concentration up to 1.5 M (not shown). One known effect of osmotic imbalance on membrane fusion events is the plasma membrane fusion reaction during mating (24). Therefore, we quantified the mating reaction of the \textit{via} mutants. After 4 h, 10% of the cells in the wt as well as the \textit{via} mutants had completed the fusion reaction as judged by the formation of fleurs-de-lille.

Many mutants of the Cvt pathway for aminopeptidase 1 are also defective in autophagy and \textit{vice versa}. Growing the mutants under autophagy-inducing conditions led to increased pApe1p transport to the vacuole in both \textit{via} mutants. This indicated specific defects in pApe1p transport along the Cvt pathway but not by autophagy. Therefore, we verified that autophagy is not impaired in \textit{via} mutants. Yeast deficient in the vacuolar endopeptidase PrA are unable to degrade the vacuolar single membrane autophagic bodies. These accumulate within the vacuoles in these cells and can be seen in phase contrast microscopy (22). Mutants deficient in autophagy and with a deleted PrA gene do not accumulate autophagic bodies. The wt strain, \textit{via10} and \textit{via14} as well as the respective strains with a deleted PrA gene (\textit{D}pep4) were grown in rich medium (YPD) to the stationary phase (Fig. 9B). Autophagic bodies could be seen in \textit{via10D}pep4 (D) and \textit{via14D}pep4 (F) as well as
The precursor form of aApe1p is proteolytically active. wt and via mutants with and without proteinase A (ΔpApe4) were grown on minimal media for 16 h, and overlay assays for aminopeptidase 1 activity were performed. Images were recorded with a CCD chemiluminescence camera system.

Do the autophagosomes transport cargo proteins? Vacular proteinase PrA is required for the maturation of several vacular hydrolases, and its deficiency leads to impaired vacular function and reduced vitality of PrA mutant strains under conditions of nutrient starvation (25). Mutants of the Cvt pathway and autophagy have a reduced viability under nitrogen starvation due to a block in cytoplasm to vacuole transport of proteins to be degraded by macro-autophagy. Vacular protein turnover and synthesis of vacular enzymes as well as proteins related to vacular function are induced in cells grown in rich media (YPD) to stationary phase (A600 > 2, glucose 0%) and to a larger extent in nitrogen-limited media (MV-D) and nitrogen starvation media (MV-N) (26). We tested via10 and via14 for defects in the transport of cytoplasmic proteins to the vacuole by following growth in these media and tested for enhancement of stress under starvation conditions by deletion of the PrA gene PEPA4. Both via mutants grew as fast as the wt strain and reached the same cell densities in rich media, but the via14 mutant showed a prolonged lag-phase after inoculating the media from a G1-arrested stationary culture in rich media (YPD) (Fig. 9C). The same result was obtained when strains were grown in rich media containing ethanol as the sole carbon source (YPE) (not shown). Under nitrogen-limiting conditions in MV-D, neither of the via mutants reached the cell densities of the wt strain (Fig. 9C), but via10 cells grew in the early log-phase as fast as the wt strain. via14 cells displayed a prolonged lag-phase also under these conditions, grew slower then the wt and the via10 cells, and did not reach the cell density of the via10 mutant. Both mutant cultures contained 20% dead cells compared with 6% in the wt culture during mid-log-phase at glucose concentrations between 1.5 and 0.5 and 40% compared with 15% in stationary cultures at 0% glucose as measured by trypsin blue staining. The increased sensitivity of the mutants to nitrogen starvation was verified by growing the cells in nitrogen starvation medium (MV-N). via14 was also able to grow under these conditions albeit at a reduced rate. Deletion of the vacular PrA prolonged the lag-phase after inoculating the cultures, but reduced the growth rates of the wtΔpApe4, via10ΔpApe4 as well as via14ΔpApe4 mutants to the same extent. The enhancement of the via mutant growth phenotype by PrA deletion indicates that via mutants are not defective in autophagocytic mechanisms and that the observed autophagic bodies contain cargo proteins delivered to the vacuole for degradation as well as pApe1p. However, the via mutants are stressed by nutrient starvation indicating that, besides pApe1p and α-mannosidase, other proteins are transported along the Cvt pathway as well.

**DISCUSSION**

pApe1p rapidly assembles with a half time of 2 min into a dodecameric complex in the cytoplasm, which is packaged into double-membrane Cvt vesicles. All pApe1p and mApe1p are in dodecameric complexes (27). Targeting of pApe1p into Cvt vesicles requires the propeptide, which consists of 45 amino acids forming a helix-turn-helix structure. Mutations of the signal sequence, which prevent helix formation, also abolish pApe1p transport but appear not to influence dodecamer formation (15, 16). The via mutants of pApe1p transport were isolated based on the absence of enzymatic activity, which requires homododecamerization of the protein. In the two complementation groups via10 and via14 pApe1p does not form homododecamers but is transported into Cvt vesicles, where it accumulates. The fraction of Ape1p transported to the vacuole is present as a homododecameric complex. The Ape1p in the mutants is sensitive toward cytoplasmic proteolysis leading to reduced pApe1p levels under conditions of Cvt vesicle dependent transport.

To test for Cvt vesicle formation, the propeptide of pApe1p fused to GFP was expressed under the APE1 promoter. This fusion protein is rapidly degraded in the cytoplasm as well as vacuoles and can therefore be used to test for Cvt vesicle formation and to demonstrate accumulated Cvt vesicles by immunofluorescence microscopy. We could not detect immunofluorescence in the cytoplasm of the via mutants. pro-GFP was degraded in all cell lines, but via mutants contained reduced amounts of the protein demonstrating impaired Cvt vesicle formation.

Transport of pApe1p to the vacuole can be up-regulated in the mutants by inducing autophagy, but this does not restore enzymatic activity and thus homododecamerization. This demonstrates a specific defect in the Cvt pathway and that doodecamerization of pApe1p is linked to the Cvt pathway. This indicates that VIA10 and VIA14 encode novel factors with chaperone-like activities required for the formation of native pApe1p homododecamers and transport by the Cvt pathway and that these factors are also required for the formation of native complexes under conditions of autophagy, but that they are not required for vacular transport of autophagosomes. The cytoplasmic HSC70 family members Ssa1p, Ssa2p, Ssa3p, and Ssa4p are required for vacular transport of pApe1p by the Cvt pathway. HSC70 mutants accumulate pApe1p in Cvt vesicles, indicating that they are required for Cvt vesicle formation. Dodecamerization of pApe1p is not disturbed, and we do not find evidence for a direct HSC70-pApe1p interaction in vivo (18). We also expressed either Ssa1p or Ssa2p from single-copy vectors in both via mutants, but this did not restore Ape1p enzymatic activity or dodecamerization.

How are dodecamerization and Cvt vesicle formation linked to each other? (i) Is no dodecamer formed if no Cvt vesicles are present? (ii) Are no Cvt vesicles formed without formation of dodecamers? or (iii) Is the same protein or protein complex required for dodecamerization as well as Cvt vesicle formation? These three questions are answered as follows. (i) This is likely to be the case, because pApe1p complexes accumulating upon overexpression of pApe1p lead to the formation of oligomers, which are not transported to the vacuole (2). (ii) Expression of pro-GFP, which does not oligomerize, is transported by the Cvt pathway demonstrating that dodecamerization is not a prerequisite for Cvt vesicle formation (23). (iii) Pro-GFP is not protected by a Cvt vesicle membrane in the via mutants, demon-

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P. V. Schu, unpublished.
Dodecamerization of pApe1p in the cytoplasm is required for the formation of enzymatically active dodecamers of aminopeptidase 1, because inducing autophagy restores pApe1p transport to the vacuole and its proteolytic processing, but this does not restore enzymatic activity. Therefore, we think that VIA10 and VIA14 are required for the controlled dodecamerization as well as Cvt vesicle formation, possibly as part of a multimeric complex. This way they can control the potentially harmful cytoplasmic proteolytic activity of pApe1p, because enzymatically active complexes are only formed, if they are readily compartmentalized. This method of controlling proteolytic activity also explains the existence of a second transport pathway for vacuolar enzymes.

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