Vps51 Is Part of the Yeast Vps Fifty-three Tethering Complex Essential for Retrograde Traffic from the Early Endosome and Cvt Vesicle Completion*

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Autophagy, pexophagy, and the Cvt pathway are processes that deliver hydrolytic enzymes and substrates to the yeast vacuole/lysosome via double-membrane cytosolic vesicles. Whereas these pathways operate under different nutritional conditions, they all employ common machinery with only a few specific factors assisting in the choice of the delivery program and the membrane source for the sequestering vesicle. We found that the YKR020c gene product is essential for Cvt vesicle formation but not for pexophagy or induction of autophagy. Autophagosomes in the ykr020wΔ mutant, however, have a reduced size. We demonstrate that Ykr020 is a subunit of the Vps fifty-three tethering complex, composed of Vps52, Vps53, and Vps54, which is required for retrograde traffic from the early endosome back to the late Golgi, and for this reason we named it Vps51. This complex participates in a fusion event together with Tgl1 and Tgl2, two SNAREs also shown to be necessary for Cvt vesicle assembly. In particular, those factors are essential to correctly target the prApe1-Cvt19-Cvt9 complex to the preautophagosomal structure, the site of Cvt vesicle formation.

Autophagy is a catabolic process conserved among yeast, plants, and animal cells that permits the cell to eliminate unwanted or unnecessary proteins and organelles and to recycle the components for reuse (1, 2). The organellar turnover is exclusively accomplished in the lysosome/vacuole lumen by a wide range of hydrolases capable of breaking down all cellular constituents (1, 2). Autophagy plays an essential role during normal physiological processes such as starvation, cellular differentiation, cell death, and aging, but also in preventing some types of cellular dysfunction including cancer (2).

Studies in the yeast Saccharomyces cerevisiae have led to the identification of a large number of molecular components that form the autophagic machinery (1). Interestingly, most of these proteins are also utilized for the cytoplasm to vacuole targeting (Cvt) pathway (3, 4), which assures the delivery of the resident vacuolar hydrolase aminopeptidase I (Ape1) (5, 6). The same components are also required for peroxisome degradation, or pexophagy (7). These various processes operate under different nutritional conditions, but biochemical and morphological analyses have shown that in all cases the cargo material (precursor Ape1 (prApe1), bulk cytoplasm or a specific organelle) is sequestered by a cytosolic double-membrane vesicle (7–11). The basic mechanism that leads to the formation of this structure, called an autophagosome, Cvt vesicle, or pexophagosome, is identical in all three pathways and it can be divided into five discrete steps: vesicle induction/nucleation, cargo selection/packaging, vesicle formation/completion, docking/fusion with the vacuole, and subvacuolar vesicle breakdown (1, 2).

In the case of pexophagy and the Cvt pathway, the cargo may be specifically targeted to the sequestering membrane where it starts to be wrapped by a double lipid bilayer. This process leads to the creation of the cytosolic double membrane vesicle. The completed vesicle docks with the lysosome/vacuole and successively fuses with it. In this way the inner vesicle is liberated into the lysosome/vacuole lumen where it is finally consumed by hydrolyases.

Cellular signals dictate the selection of the cargo material but also the size of the forming vesicle (9, 12, 13). The serine/threonine protein kinase Apg1 and its interacting partner Apg13 are two components that play a part in all three pathways. These proteins seem to have a central role in determining the specific cellular response to nutrient conditions (4, 7, 13–15). Phosphorylation and dephosphorylation reactions mediate the association of Apg1 and Apg13 (13) creating a modular core complex able to interact with factors such as Apg17, Cvt9, and Vac8 that are specific only for one or two pathways (13, 16–18) (Table II).

The rest of the components involved in the biogenesis of autophagosomes and Cvt vesicles include two conjugation systems that lead to the covalent linkage of the ubiquitin-like protein Aut7 to a molecule of phosphatidylethanolamine and the formation of a multimeric complex composed of Apg12-Apg5 and Apg16 (19). In addition, an autophagy-specific phosphatidylinositol (PtdIns) 3-kinase complex is involved in the synthesis of PtdIns(3)P that may serve to recruit downstream effectors that function in autophagy and the Cvt pathway (1, 20–23).

These shared factors and all the regulatory elements localize to a punctate perivacuolar organelle, also called the preautophagosomal structure (PAS), that is believed to be the formation structure; PtdIns, phosphatidylinositol; tSNARE, target soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor; VPT, Vps fifty-three; vSNARE, vesicle SNARE; YFP, yellow fluorescent protein; SMD, synthetic minimal medium; MES, 4-morpholineethanesulfonic acid.

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tion site of autophagosomes and Cvt vesicles (24–26). Most of the autophagy (Apg)/Cvt proteins are cytosolic and achieve their correct localization by interaction with other factors or by specific binding to lipids such as phosphatidylethanolamine or PtdIns(3)P (29). That seems to correlate with studies in the Cvt pathway differ at least in part. For example, Aut7 is specific binding to lipids such as phosphatidylethanolamine or their correct localization by interaction with other factors or by the autophagy (Apg)/Cvt proteins are cytosolic and achieve the PAS.

For the BY4742 background used in this study (27), the MATa leu2–3,112 ura3–52 trp1 pho8 was replaced with either the URA3 gene from the 3’ fragment into a pRS414 plasmid (36) and cloned into the EcoRI site of pRS416-CuProtA vector (25) behind sequences expressing two IgG binding domains of protein A (PA) and the CFP promoter, and before the CYC1 terminator. The new plasmid was called pCuPAYKR020(416). This construction was also transferred as a Kpn1-SacI fragment into a pRS414 plasmid (36) creating pCuPAYKR020(414). All enzymes for manipulation of DNA were from New England Biolabs (Beverly, MA). Plasmids expressing PA (pRS416-CuProtA), GFP-Sn1 (pGFP14), GFP-Apel (pTS466), GFP-Apel (pTS470), Cvt19-CFP (pCVT19CFP(414)), GFP-Apel (pRS416YFP-Apel(7)), CFP-Apel (pPS97), and CFP-Cvt9 (pPS98) have been described elsewhere (24, 25, 37, 38).

**Protein Extraction and Western Blot**—Cells were loaded on SDS-PAGE gels and after Western blotting, membranes were probed with anti-Apel monoclonal antiserum (6).

**Fluorescence Microscopy**—Cells grown to early logarithmic (log) phase in SMD medium or shifted to SD-N medium for 3 h. 1 A600 unit of cells was collected by centrifugation and proteins were precipitated with 500 μl of ice-cold 10% trichloroacetic acid for 30 min. After spinning the samples for 5 min, pellets were washed once with acetone. Pellets were air dried, resuspended in 100 μl of MURB buffer (50 mM NaHPO4, 25 mM MES, pH 7.0, 1% SDS, 3 mM urea, 0.5% 2-mercaptoethanol, 1 mM NaN3, and 2% glucose), and heated at 75 °C for 10 min. Aliquots of 10 μl were loaded on 8% SDS-PAGE gels and after Western blotting, membranes were probed with anti-Apel monoclonal antiserum (6).

**Strains and Growth Media**—The S. cerevisiae knockout strains in the BY4742 background used in this study (28) were grown in SD medium for 3 h. 1 A600 unit of cells was collected by centrifugation and proteins were precipitated with 500 μl of ice-cold 10% trichloroacetic acid for 30 min. After spinning the samples for 5 min, pellets were washed once with acetone. Pellets were air dried, resuspended in 100 μl of MURB buffer (50 mM NaHPO4, 25 mM MES, pH 7.0, 1% SDS, 3 mM urea, 0.5% 2-mercaptoethanol, 1 mM NaN3, and 2% glucose), and heated at 75 °C for 10 min. Aliquots of 10 μl were loaded on 8% SDS-PAGE gels and after Western blotting, membranes were probed with anti-Apel monoclonal antiserum (6).

**Fluorescence Microscopy**—Cells grown to early logarithmic (log) phase in SMD medium or shifted to SD-N medium for 3 h, were prepared for fluorescence and stained with FM 4-64 (Molecular Probes, Eugene, OR) as described previously (39). Fluorescence signals were viewed in the use of a Nikon E-800 fluorescent microscope (Mager Scientific, Dexter, MI). The images were captured with an ORCA II CCD camera (Hamamatsu, Bridgewater, NJ) with the use of Openlab software (Im-precision, Lexington, MA).

**REFERENCES**

**Figures**

1. **Fig. 1**. Microscopy images of Cvt and autophagy vesicles in MATa BY4742 cells. A, C, and E, control cultures; B, D, and F, cultures starved for nitrogen in SD medium. The cells were stained with FM 4-64 and probed with anti-Ape1 polyclonal antiserum (6). 2. **Fig. 2**. Western blot of total cellular extracts probed with anti-Ape1 polyclonal antiserum (6).

**TABLE I**

| Strain          | Genotype                  | Source          |
|-----------------|---------------------------|-----------------|
| BY4742          | MATa his3Δ1 leu2Δ10 lys2Δ10 ura3Δ10 | ResGen™        |
| SEY6210         | MATa his3Δ200 leu2Δ3,112 lys2Δ30–801 ura3Δ3–52 trpl1Δ2001 suc2Δ30 | Ref. 49       |
| TN124           | MATa leu2Δ3,112 ura3Δ3–52 trpl1Δ pho8Δ pho8AΔ60 pho13Δ trpl1Δ LEU2 | Ref. 45       |
| DYG103          | TN124 apg9Δ3::URA3         | Ref. 17        |
| FYR107          | MATa his3Δ1 leu2Δ10 lys2Δ10 ura3Δ10 vps53Δ::kanMX4 | This study     |
| FYR116          | SEY6210 VPS53::3xHA::TRP1 vps52Δ::URA3 K1. LoxP | This study     |
| FYR117          | SEY6210 VPS53::3xHA::TRP1 ykr020Δ::URA3 K1. LoxP | This study     |
| FYR118          | SEY6210 YKR020Δ-13xMyc::TRP1 | This study     |
| FYR119          | BY4742 vps52Δ::kanMX4 vac8Δ::URA3 | This study     |
| FYR121          | TN124 ykr020Δ::URA3 K1. LoxP | This study     |
| FYR122          | TN124 vps52Δ::URA3 K1. LoxP | This study     |
| FYR123          | SEY6210 vps53Δ::LEU2 ykr020Δ::HIS5 S.p. LoxP + pVAM3-6.414 | This study     |
| FYR124          | SEY6210 vps53Δ::LEU2 ykr020Δ::HIS5 S.p. LoxP + pVAM3-6.414 | This study     |
| FYR125          | SEY6210 vps53Δ::LEU2 vps52Δ::URA3 K1. LoxP + pVAM3-6.414 | This study     |
| FYR126          | SEY6210 vps52Δ::URA3 K1. LoxP | This study     |
| FYR113          | SEY6210 vps53Δ::HIS5 S.p. | This study     |
| FYR118          | SEY6210 VPS53–3xHA::TRP1 | This study     |
| FYR119          | SEY6210 VPS53–3xHA::TRP1 | This study     |
| FYR120          | SEY6210 VPS54–3xHA::HIS5 S.p. | This study     |
| FYR118          | SEY6210 YKR020Δ-13xMyc::TRP1 vps52Δ2::URA3 K1. LoxP | This study     |
| TDY2            | SEY6210 vps53Δ::LEU2 + pVAM3-6.414 | Ref. 57       |
Protein A Affinity Isolation—Cells were first grown overnight in SMD medium, then diluted with YPD and grown for an additional 5 hours. 50 A_{600} units of cells were harvested, converted to spheroplasts, and kept frozen. Spheroplasts were resuspended and Dounce homogenized in 2 ml of lysis buffer (40) containing 2 mM phenylmethylsulfonyl fluoride. Cell lysates were then centrifuged at 15,000 g for 15 min and 1.6 ml of supernatant was incubated for 2 h at 4°C with 20 µl of prewashed IgG Sepharose beads (Amersham Biosciences). Beads were then washed twice with lysis buffer (40), once with lysis buffer containing 300 mM KCl, once with lysis buffer containing 500 mM KCl, then once again with lysis buffer containing 300 mM KCl, and finally 3 times with the initial buffer. Finally, beads were heated at 75°C for 10 min in 50 µl of MURB buffer. After SDS-PAGE and Western blot, membranes were probed with anti-HA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

For cross-linking experiments, spheroplasts were prepared as above but not frozen. Instead, they were resuspended in 200 µl of phosphate buffer (25 mM potassium phosphate, pH 7.4, 200 mM sorbitol, 20 mM phenylmethylsulfonyl fluoride, 10× Complete EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN)) containing 1.5 mM dithiothreitol (sucinimidyl propionate) (Pierce). Suspensions were incubated for 30 min at room temperature. To quench the cross-linker, 200 µl of ice-cold 200 mM Tris-HCl (pH 7.4) were added and tubes were transferred to 4°C for 5 min. Finally, 1.6 ml of ice-cold dilution solution (187.5 mM KCl, 6.25 mM MgCl₂, 1.25% Triton X-100) was added and after Dounce homogenization, protein A affinity isolation was performed as above. After SDS-PAGE and Western blot, membranes were probed with anti-HA, anti-Tlg1 (41), anti-Pep12 (Molecular Probes), or anti-Sed5 (42) antisera or antibodies.

Miscellaneous Procedures—The analyses of protease sensitivity, Prc1 missorting, cell viability under nitrogen starvation conditions, pexophagy, Pho80p activity, and electron microscopy using Spurr’s resin (Ted Pella, Redding, CA) for embedding were conducted as described previously (7, 23, 27, 43).

RESULTS

The ykr020wΔ, vps52Δ, vps53Δ, and vps54Δ Mutants Block Precursor Ape1 Maturation Only Under Rich Growth Conditions—We identified the YKR020w gene in a genomic approach where nonessential gene deletions in the yeast S. cerevisiae were scored for a defect in precursor Ape1 (prApe1) maturation. The ykr020wΔ cells grown in rich medium accumulate prApe1, indicating a defect in the Cvt pathway (Fig. 1A). Certain sgg and cvt mutants that are defective in prApe1 transport under vegetative conditions are able to import the protein into the vacuole when autophagy is induced by starvation. For example, mutants specific for the Cvt pathway such as cvtθ, vac8, cvt13, and cvt20 or mutants that block autophagosomal expansion but not induction such as aux7 are able to mature prApe1 when shifted to starvation conditions (12, 16, 17, 23). We examined processing of prApe1 in the ykr020wΔ mutant following induction of autophagy. The ykr020wΔ strain displayed normal processing of prApe1 when this strain was shifted to a nitrogen starvation medium (Fig. 1A), indicating that autophagic induction is not impaired.

A genome-wide approach for the identification of yeast protein complexes by mass spectrometry indicated a putative interaction between Ykr020p and Vps52/Scac2 (44). Vps52 together with Vps53 and Vps54 form the so-called VFT complex, a putative tethering factor required for the retrieval of proteins from the early endosome to the late Golgi (33, 45). The vps52Δ and vps54Δ mutants were also identified in our screen that detected the prApe1 defect in the ykr020wΔ strain. Furthermore, the vps52Δ and vps54Δ cells displayed a similar property in reverting the prApe1 maturation defect after nitrogen starvation (Fig. 1A). In contrast, vps53Δ cells showed normal prApe1 processing in all media. However, further analysis of the commercial vps53Δ haploid strain supplied by ResGen indicated that the deleted gene was not VPS33. To analyze the prApe1 phenotype in the vps53Δ strain, we sporulated the ResGen vps53Δ homozygous diploid strain. The resulting haploid spores showed the same reversible block in prApe1 maturation that was observed for the ykr020wΔ, vps52Δ, and vps54Δ strains (Fig. 1A).

A defect in prApe1 maturation under vegetative conditions can be caused either by a block in the Cvt pathway or by inefficient cleavage of the prApe1 propeptide by proteases after reaching the vacuole lumen. The vps52Δ, vps53Δ, and vps54Δ mutants missort carboxypeptidase Y (Prc1) (46, 47). This mis-sorting is caused by a block in retrieval of Vps10 to the Golgi complex (46); Vps10 is the receptor required for Prc1 transport to the vacuole (48). We examined the sorting of Prc1 in the ykr020wΔ mutant to determine whether it displayed a similar phenotype. Cells from the ykr020wΔ strain were converted to spheroplasts, subjected to pulse-chase labeling, and immunoprecipitated with antiserum to Prc1. In wild type cells, Prc1 was not correctly delivered to the vacuole and processed to the mature form; essentially no missorting was observed (Fig. 1B). In agreement with the published data, vps52Δ cells secreted part of Prc1 as the p2 Golgi-modified form (Fig. 1B) (46). The result obtained with ykr020wΔ cells was very similar to that seen with the vps52Δ strain (Fig. 1B).

Most vacuolar protein sorting (vps) mutants display pleiotropic defects in the sorting of additional vacuolar hydrolases including Pep4 and Prb1 (49–51). As a result, these strains are severely compromised for vacuolar processing activity. Accordingly, we could not exclude a defect in prApe1 processing because of reduced proteolytic activity of the vacuole. To explore this possibility, we took advantage of a green fluorescent protein (GFP)-tagged version of prApe1 that can be used to monitor defects in the Cvtp pathway (38). In wild type cells expressing GFP-Ape1, fluorescence was localized to the vacuole lumen either when cells were grown in rich medium or after nitrogen starvation (Fig. 1C). The vps52Δ, vps53Δ, vps54Δ, and ykr020wΔ strains have fragmented vacuoles (46, 47) (Fig. 4). Nonetheless, in contrast to the wild type strain, we could determine that when vps52Δ, vps53Δ, vps54Δ, and ykr020wΔ cells expressing the same GFP-Ape1 chimera were grown in rich medium, GFP-Ape1 was concentrated at a perivacuolar punctate structure (Fig. 1C). This site may correspond to the PAS (24, 25). The PAS is generally enhanced in mutants with a defect either in the formation of Cvtp vesicles or in their fusion with the vacuole (24, 25). This result indicates that the missing of vacuolar proteases in the vps52Δ, vps53Δ, vps54Δ, and ykr020wΔ strains does not account for the accumulation of prApe1. Rather, this defect is because of a block of prApe1 import into the vacuole lumen. Transport of GFP-Ape1 into the vacuole lumen could be restored after transferring the same cells into medium lacking nitrogen (Fig. 1C), in agreement with the reversibility of the prApe1 processing phenotype.

With the previous analysis we could not differentiate between a block in Cvtp vesicle formation or fusion of completed vesicles with the vacuole. This question was addressed with a protease sensitivity experiment. Yeast cells were converted to spheroplasts and lysed under conditions that allow lysis of the plasma membrane while retaining the integrity of subcellular compartments (52). The lysates were then treated with proteinase K in the absence or presence of detergent. During Cvtp vesicle formation, prApe1 is not yet enclosed within a membrane compartment and consequently, in mutants affecting this process, prApe1 is accessible to exogenous proteases. In strains such as ccz1Δ that have a block in fusion (53, 54), prApe1 is enwrapped by the membrane of the Cvtp vesicle and therefore isolated from the cytosol. As shown in Fig. 1D, in ccz1Δ cells prApe1 was protected from proteinase K. In contrast, prApe1 was fully accessible to the proteolytic action of the same proteasine in the vps52Δ strain. Identical results were also obtained with vps54Δ and ykr020wΔ cells (data not
A

B

C

D

Fig. 1. The \textit{ykr020wΔ}, \textit{vps52Δ}, \textit{vps53Δ}, and \textit{vps54Δ} cells have a defect in Cvt vesicle completion. \textit{A}, The \textit{ykr020wΔ}, \textit{vps52Δ}, \textit{vps53Δ}, and \textit{vps54Δ} strains have a reversible inhibition of prApe1 processing similar to that of \textit{vac8Δ} cells. Wild type (\textit{WT}), \textit{ykr020wΔ}, \textit{vps52Δ}, \textit{vps53Δ} (\textit{FRY107}), \textit{vps54Δ}, \textit{apg9Δ}, and \textit{vac8Δ} cells in the \textit{BY4742} background grown either in \textit{YPD} or nitrogen starved in \textit{SD-N} medium for 4 h were trichloroacetic acid precipitated. Acetone-washed proteins were then resolved by SDS-PAGE and prApe1 maturation analyzed by immunoblot. \textit{B}, Prcl is mislocalized to the periplasmic space in the \textit{ykr020wΔ} mutant similarly to \textit{vps52Δ} cells. Cells from wild type, \textit{ykr020wΔ}, and \textit{vps52Δ} strains in the \textit{BY4742} background were transformed with a plasmid expressing the N-terminal GFP-tagged prApe1 (pTS446). Transformed cells were grown either in SMD or nitrogen starved for 3 h in SD-N medium and examined with a fluorescence microscope. DIC, differential interference contrast. \textit{D}, precursor Ape1 is protease-sensitive in the \textit{vps52Δ} mutant.

We concluded that the \textit{vps52Δ}, \textit{vps54Δ}, and \textit{ykr020wΔ} mutants, and by extension the \textit{vps53Δ} mutant, are blocked at the formation/completion step of Cvt vesicle biogenesis. Ykr020, Vps32, Vps33, and Vps54 Are Essential during Starvation but Not Required for Autophagy and Pexophagy—Most of the machinery required for the Cvt pathway is also exploited by autophagy and pexophagy (3, 4, 7). Results shown in Fig. 1, \textit{A} and \textit{B}, indicated that prApe1 processing was normal in the \textit{vps52Δ}, \textit{vps53Δ}, \textit{vps54Δ}, and \textit{ykr020wΔ} strains when autophagy was induced by nitrogen starvation. Recently, Ishihara et al. (29) reported that the Cvt pathway is operative and autophagy blocked during nitrogen deprivation in a sec12 mutant. Accordingly, we could not exclude the possibility that prApe1 maturation in the \textit{vps52Δ}, \textit{vps53Δ}, \textit{vps54Δ}, and \textit{ykr020wΔ} strains under starvation conditions was because of the Cvt pathway. To explore this possibility, we took advantage of \textit{vac8Δ}, a mutant that is defective for the Cvt pathway but not for autophagy (17). We made a \textit{vps52Δ vac8Δ} double mutant to see if prApe1 transport still occurred when those cells were starved for nitrogen. Precursor Ape1 processing was analyzed by Western blot in wild type, \textit{vps52Δ}, \textit{vac8Δ}, and \textit{vps52Δ vac8Δ} cells grown either in the presence or absence of nitrogen. As expected, in both \textit{vps52Δ} and \textit{vac8Δ} cells prApe1 transport was blocked when the strain was grown in rich medium and restored when cells were shifted to a medium lacking nitrogen (17) (Fig. 2A). A similar result was obtained with the \textit{vps52Δ vac8Δ} double mutant (Fig. 2A). The ability to transport prApe1 to the vacuole under nitrogen starvation conditions in the absence of \textit{Vac8} indicated that in \textit{vps52Δ} cells this process was performed by autophagy and not by the Cvt pathway.

The observation that prApe1 import in the \textit{vps52Δ} strain is dependent on autophagy cannot be interpreted as indicating that autophagy is fully functional in this strain; prApe1 can be transported to the vacuole in an autophagy-dependent process even in situations where autophagy is severely impaired (12, 17). For example, in the absence of \textit{Atg7}, induction of autophagy leads to the formation of abnormally small autophagosomes that can transport prApe1 to the vacuole but that cannot maintain a normal level of autophagy (12). We examined survival of the \textit{vps52Δ}, \textit{vps53Δ}, \textit{vps54Δ}, and \textit{ykr020wΔ} strains under starvation conditions as another method for assessing the autophagic capacity of these mutants. Strains that are defective in autophagy display limited viability under starvation conditions (55). The wild type, \textit{apg1Δ} (15), \textit{cvt9Δ} (16), \textit{vps32Δ}, \textit{vps33Δ}, \textit{vps43Δ}, and \textit{ykr020wΔ} strains were grown to mid-log phase, shifted to nitrogen starvation conditions, and their viability was determined over time. The wild type strain survived nitrogen starvation for more than 12 days without a significant decrease in viability (Fig. 2B). The \textit{cvt9Δ} strain, defective primarily in the Cvt pathway, was also relatively
Cvt22 Is Part of the VFT Complex

The starvation sensitivity suggests that the vps52Δ, vps53Δ, vps54Δ, and ykr020wΔ strains may be partially defective in autophagy. Alternatively, this phenotype could be because of other defects. Under conditions of nutrient stress it becomes necessary for the cell to transport cytoplasm to the vacuole by autophagy. However, subsequent to vacuolar delivery, these components must be degraded to generate an internal supply of nutrients (8). Cells require a fully functional vacuole to degrade and transport the recycled material back to the cytosol. The vps52Δ, vps53Δ, vps54Δ, and ykr020wΔ mutants affect the proper delivery of resident vacuolar hydrolases (46, 47) (Fig. 1). For this reason, it could not be excluded that the loss of viability observed after nitrogen starvation was caused by the inability to degrade the cytoplasmic substrates that had been delivered through autophagy. To directly quantify autophagy, we decided to measure the vacuolar processing of the cytosolic marker protein Pho8Δ60. This truncated form of the vacuolar alkaline phosphatase (Pho8) lacks the transmembrane domain and consequently localizes to the cytosol (56). This protein is delivered to the vacuole exclusively by autophagy. Proteolytic cleavage of the Pho8Δ60 propeptide in the vacuole lumen generates the active form of the enzyme, which can be detected by an activity assay (56). The YKR020wΔ and YPS52 genes were knocked out in a strain where the chromosomal PHO8 gene was replaced with pho8Δ60, and phosphatase activity was determined either before or after nitrogen starvation. In wild type cells, there was low alkaline phosphatase activity when cells were grown in rich medium (Fig. 2C). There was an ~7-fold increase in activity following the induction of autophagy when cells were shifted to SD-N. Apg13 is essential for autophagy (14, 17). In contrast to wild type cells, there was no increase in alkaline phosphatase activity from Pho8Δ60 when apg13Δ cells were shifted to starvation conditions. As shown in Fig. 2C, autophagy in the ykr020wΔ and vps52Δ strains was reduced to 75 and 60%, respectively, of the wild type levels but was not completely abolished compared with apg13Δ cells. This result indicates that autophagy was at least partially active in the vps52Δ and ykr020wΔ mutants.

There are two explanations for the reduced autophagic activity in the vps52Δ and ykr020wΔ strains. First, these differences may reflect a slower cellular metabolism and as a result, deletion strains form a reduced number of autophagosomes compared with wild type cells during the same time period. In fact, the vps52Δ, vps53Δ, and vps54Δ strains grow substantially slower than wild type cells, whereas the ykr020wΔ mutant has an intermediate doubling rate (data not shown). The second possibility is that in the mutant cells, autophagosomes are smaller and consequently they are able to transport less material into the vacuole. Accordingly, we decided to analyze the morphology of the autophagosomes in the mutant strains by electron microscopy to determine their size and number. Autophagosomes are transient structures that fuse with the vacuole. To stabilize cytosolic autophagosomes we took advantage of a conditional allele of VAM3, a gene coding for a tSNARE required for the fusion of Cvt vesicles and autophagosomes.
viability very quickly, similar to what was observed for Kaiser and Schekman (78). Uranyl acetate-stained sections were ob-
fixation, dehydration, and embedding were carried out as described by SD-N medium. Cells were then grown at 37°C.
FRY125 cells were grown in YPD medium at 26°C to early log phase. Cultures were split in half and centrifuged. One sample was resus-
pended again in YPD medium whereas the other was resuspended in SD-N medium. Cells were then grown at 37°C for 3 h. Permanganate fixa-
tion, dehydration, and embedding were carried out as described by Kaiser and Schekman (78). Uranyl acetate-stained sections were ob-
erved using a Philips CM10 transmission electron microscope. Examples of autophagosomes are indicated with an arrow. The bar is 1 μm.

Fig. 3. The ykr020αΔ and vps52Δ strains have smaller autophagosomes. Wild type (WT, TDY2), ykr020αΔ (FRY124), and vps52Δ (FRY125) cells were grown in YPD medium at 26°C to early log phase. Cultures were split in half and centrifuged. One sample was resus-
pended again in YPD medium whereas the other was resuspended in SD-N medium. All three strains showed the absence of autophagosomes when grown in rich medium (Fig. 3). Under nitrogen starvation conditions, however, vps52Δ and ykr020αΔ cells accumulated smaller autophagosomes than those present in the wild type strain (Fig. 3). The total number of autophagosomes in the mutant strains, however, appeared comparable with that in the wild type strain. We therefore concluded that reduced autophagic activity in the ykr020αΔ, vps52Δ, vps53Δ, and vps54Δ strains is caused by the inability to assemble normal sized autophagosomes.

Mutations in structural genes encoding vacuolar hydrolases such as PEP4 and PRB1 result in a starvation-sensitive phenotype (8, 58). This finding is in agreement with the requirement for Prb1 (protease B) in the breakdown of autophagic bodies, the single membrane subvacuolar vesicles that contain the cytoplasmic cargo resulting from autophagy (8). To our knowledge, however, the viability of vps mutants that are blocked in the biosynthetic delivery of resident hydrolases to the vacuole has not been examined in starvation conditions. For this reason, we decided to analyze the viability under starvation conditions of other deletion strains with a vps defect.

We selected four strains that affect different functions of the late endosome without interfering with normal prApe1 trans-
port.2 The vps4Δ, vps5Δ, vps27Δ, and vps29Δ cells all lost viability very quickly, similar to what was observed for vps52Δ.

2 F. Reggiori and D. J. Klionsky, unpublished observations.

vps53Δ, vps54Δ, and ykr020αΔ cells (Fig. 2D). This result confirmed our hypothesis that normal traffic to the vacuole is required to utilize the potential nutrient pool created by autophagy and provides a likely explanation for the starvation sensitivity of strains such as vps52Δ, vps53Δ, vps54Δ, and ykr020αΔ that do not appear to be defective for autophagy.

When cells are shifted from conditions that necessitate peroxisome function (e.g. oleic acid) to glucose, excess peroxisomes are degraded (7, 59, 60). Peroxisome degradation requires most of the machinery that is needed for autophagy (7). The specific degradation of peroxisomes, pexophagy, induced by glucose adaptation or nitrogen starvation can be monitored by follow-
ing the degradation of the peroxisomal matrix protein Fox3 (7). Growing cells were first shifted for 12 h to a medium containing glycerol, a suboptimal carbon source for yeast. Peroxisome prolif-
eration was then induced by transferring cells for 19 h into a medium with oleic acid as the sole carbon source. Once shifted to SD-N, the excess peroxisomes were delivered to the vacuole and degraded in wild type cells (Fig. 2E). Pexophagy is blocked in mutants such as apg1Δ where autophagy is defective, and consequently Fox3 levels remain unchanged (7) (Fig. 2E). Fox3 degradation in ykr020αΔ, vps52Δ, vps53Δ, and by extension vps54Δ cells was essentially normal with only some minor differences in the degradation rate relative to wild type cells (Fig. 2E). We concluded that the VFT complex and Ykr020 are not required for pexophagy.

Sncl Recycling Is Blocked in ykr020αΔ, vps52Δ, vps53Δ, and vps54Δ Cells—Vps52, Vps53, and Vps54 are subunits of the VFT complex and in their absence the transport step between the early endosome and late Golgi is blocked (40). Sncl is a member of the vSNARE family and it mediates fusion of exocy-
tic vesicles with the plasma membrane (37, 61). Sncl is mostly at the cell surface, but it undergoes rapid endocytosis and is transported from the early endosome to the late Golgi where it is reused (37). Mutants that affect early endosome function and transport from this compartment mislocalize Sncl (37, 40, 45, 62, 63). We transformed wild type, vps52Δ, vps53Δ, vps54Δ, and ykr020αΔ cells with GFP-Sncl (37) to see if the cycling of this chimera was affected. Cells were also stained with FM 4-64, a lipophilic fluorescent dye that allows visualization of the yeast vacuole (64).

As previously reported, in wild type cells most of the fluores-
cent staining corresponding to GFP-Sncl was on the plasma membrane (Fig. 4) (37). A fraction of GFP-Sncl was also observed in internal cell structures, probably organelles that Sncl passes through during its recycling pathway (Fig. 4) (37). In contrast, in the four mutants analyzed, GFP-Sncl was no longer found on the cell surface. Because of the co-localization with FM 4-64, it became evident that Sncl was mislocalized to the vacuole (Fig. 4). It should be noted that in cells where the GFP-Sncl levels were lower, the staining pattern was primarily cytosolic punctate dots. These dots may represent vesicles that cannot fuse with the late Golgi compartment; in the ab-
sence of the VFT complex and Ykr020, Sncl retrieval to the Golgi complex is blocked resulting in its delivery to the vacuole via vesicular intermediates. This cytosolic staining pattern is most evident in the vps52Δ and vps54Δ strains (Fig. 4). Default delivery of Sncl to the vacuole is similar to the fate observed for the Vps10 receptor in VFT mutant cells (46).

Another similarity between ykr020αΔ cells and VFT com-
plex mutants is the structure of the vacuole. Wild type cells typically display a single large vacuole or a multilobed org-
nelle depending on osmotic conditions (65). In contrast, this organelle is highly fragmented in vps52Δ, vps53Δ, and vps54Δ cells (46, 47). FM 4-64 staining and differential interference contrast microscopy along with electron microscopy confirmed...
the vacuole.

The fragments vacuole morphology of the vps2Δ, vps53Δ, and vps54Δ strains and showed that ykr020Δ cells have a similar vacuole morphology but with a slightly less severe fragmentation (Figs. 3 and 4). Taken together with the analysis of Prc1 sorting, these data indicate that the ykr020Δ cells have essentially identical trafficking defects as vps2Δ, vps53Δ, and vps54Δ mutants in regard to either to PrApe1 transport or retrieval from the early endosome.

Ykr020 Is Part of the VFT Complex but It Is Not Required for Its Stability—The similar trafficking and vacuole morphology phenotypes suggested that Ykr020 might be involved in the same transport step as the VFT complex. In addition, a recent proteomic analysis indicates that Ykr020 directly interacts with Vps51 (44). We decided to examine if Ykr020 was part of the VFT complex. To do so, we fused PA with the N terminus of Ykr020 (Fig. 4) and displayed more rapid pexophagy (Fig. 2 A and C). Accordingly, it seemed possible that Ykr020 was dispensable for the stability of the other components of the VFT complex. To pull down Myc-tagged Vps51 indicating that there is only one Ykr020 molecule per VFT complex. The experiment performed in panel A was repeated with a strain (PSY116) expressing Ykr020-Myc, C, Ykr020 is not required for the stability of Vps51. Wild type (WT, PSY119), vps52Δ (FRY116), and vps53Δ (FRY117) strains expressing Ykr020-Myc were grown to early log phase and proteins were precipitated with trichloroacetic acid. Proteins were separated by SDS-PAGE and analyzed by Western blot with anti-HA antibodies. Pkg1 was used to verify that the same amount of material was loaded on each gel lane. D, Vps52 is not necessary to maintain normal cellular levels of Ykr020. Wild type (WT, PSY116) and vps52Δ (FRY118) cells expressing Ykr020-Myc were analyzed as in panel C using anti-Myc antibodies.

Ykr020 is only one Ykr020 molecule per VFT complex. We repeated the PA-Vps51 affinity isolation experiment using a strain carrying a copy of the VPS51 gene tagged on its C terminus with the Myc epitope. PA-Vps51 was unable to pull down Myc-tagged Vps51 indicating that there is only one Vps51 subunit per VFT complex (Fig. 5B).

Vps52, Vps53, and Vps54 depend on each other for stability. That is, in the absence of one of those proteins, the other two are rapidly degraded (46). During our experiments, we noticed that several phenotypes associated with the loss of the VFT complex were less prominent in the vps51Δ strain. For example, the vps51Δ cells grew better, had less fragmented vacuoles (Fig. 4), survived longer in media lacking nitrogen (Fig. 2 A), and displayed more rapid pexophagy (Fig. 2C). Accordingly, it seemed possible that Vps51 was dispensable for the stability of the other subunits; in the absence of Vps51, the VFT complex was still able to have a residual activity. To investigate this hypothesis, we disrupted either VPS51 or VPS52 in the strain carrying HA-tagged Vps53. As shown in Fig. 5C, Vps53 levels were dramatically decreased in vps52Δ cells whereas they remained unchanged in the vps51Δ strain. We also decided to analyze the stability of Vps51 in cells lacking one of the three other components. To carry out this analysis we deleted the VPS52 gene in the strain expressing Myc-tagged Vps51. In the absence of Vps52, Vps51 levels were unchanged compared with wild type cells (Fig. 5D). We concluded that Vps51 is not necessary for the stability of the other components of the VFT complex.
Complex and conversely, the other VFT components are not required for maintaining the appropriate cellular levels of Vps51.

There Is a Single VFT Complex Interacting with Tlg1.—It has been shown that the VFT complex transitionally binds the vSNARE Tlg1 during the docking of early endosome-derived vesicles with the late Golgi (45). At present, we could not exclude the presence of two VFT complexes, only one of which contains Vps51. We decided to examine the interaction of the VFT complex containing Vps51 with Tlg1 to determine whether it was functioning similar to the previously analyzed VFT complex (45). Spheroplasts obtained from the strain expressing either PA-Vps51 or Vps52-HA were incubated for 30 min at room temperature in the presence of the amine-reactive cross-linker dithiobis(succinimidyl propionate) (DSP), detergent solubilized, and the PA fusions were affinity purified on IgG-Sepharose as described in the legend to Fig. 5A. Samples from the extracts (Ext) or purified fractions (IP) were analyzed by Western blot using anti-HA, Tlg1, Sed5, and Pep12 antibodies or antiserum. A longer film exposure is also shown to demonstrate the total absence of cross-linking between the Pep12 antibodies or antiserum. A longer film exposure is also shown to demonstrate the total absence of cross-linking. As can be seen in Fig. 6A, Tlg1 bound the Vps51-containing VFT complex under conditions where the other tSNAREs were not cross-linked.

Pulse-chase radiolabeling followed by Apel immunoprecipitation has shown that under rich growth conditions, Tlg2, Tlg1, and the Sec1 homologue, Vps45, are essential for the formation of Cvts vesicles but not for autophagosome biogenesis (31). This type of experiment demonstrates that these proteins have a direct impact on the vesicle formation process. To show that the steady state conditions used for our investigations were genuinely representative of a direct role of the VFT complex in Cvts vesicle biogenesis, we decided to examine the state of Apel1 in tlg2Δ cells. As expected, prApel processing was blocked when tlg2Δ cells were grown in rich medium and was restored after nitrogen starvation (Fig. 6B). This result was confirmed by analyzing the GFP-Apel1 chimera in the same cells under the same growth conditions (Fig. 8C). These results are identical to those that we obtained with the VFT complex components (Fig. 1, A and C). Because Tlg1 and Tlg2 also interact with the VFT complex (45) (Fig. 6A), it is reasonable to assume that all of these proteins are involved in the same fusion event essential for Cvts vesicle biogenesis.

Cvt9 Is Mislocalized and the Apel1-Cvt19 Complex Is No Longer Correctly Transported to the PAS in the Absence of the VFT Proteins.—Most of the Cvts and autophagy pathway components are required for the vesicle formation/completion step and localize to a perivacuolar punctate structure (24, 25). Cells lacking the VFT complex are defective in Cvts vesicle biogenesis (Fig. 1D) indicating that probably one or more proteins necessary for this process are missing. To explore this possibility, we checked if the localization pattern of GFP-Apel1-Cvt19 was altered in vps51Δ and vps52Δ cells. Of the different proteins analyzed, only Cvt9, a protein specific for the Cvts pathway and pexophagy (16), showed a different cellular distribution. Wild type cells transformed with a plasmid expressing a GFP-Cvt9 chimera under the control of the strong CUP1 promoter (68) showed GFP fluorescence at a perivacuolar punctate structure (Fig. 7A). In contrast, vps51Δ cells carrying the same construct displayed the presence of several dispersed fluorescent dots (Fig. 7A). This pattern was because of the absence of Vps51 because when the same cells were transformed with a plasmid expressing the PA-Vps51 fusion, the correct Cvt9 localization was restored (Fig. 7A). This phenotype was not caused by the overexpression of the GFP-Cvt9 chimera by the CUP1 promoter because the same fusion under the control of the endogeneous Cvts9 promoter gave identical results although the fluorescent signal was fainter (Fig. 7A). After shifting cells for 3 h to a medium lacking nitrogen, GFP-Cvt9 became a single fluorescent spot in the vps51Δ strain (Fig. 7B). Thus, starvation conditions that bypass the prApel1 block in VFT complex mutants were able to induce the correct targeting of Cvt9. The GFP-Cvt9 chimera displayed similar patterns in vps52Δ, vps53Δ, vps54Δ, and tlg2Δ strains (data not shown).

After synthesis, prApel forms a large cytosolic oligomer that binds Cvt19 (69, 70). This association triggers the transport of this complex to the site of Cvts vesicle formation in a process that requires Cvt9 (38). Our examination of GFP-tagged Apel/Cvt proteins in vps51Δ and vps52Δ cells indicated that prApel1 and Cvt19 were restricted to a single punctate structure (Fig. 1C and data not shown). However, this observation could not rule out the possibility that the fluorescent structure observed with GFP-Apel1 or GFP-Cvt19 was not correctly targeted to the site of Cvts vesicle formation, especially considering the observation that Cvt9 is mislocalized in vps51Δ and VFT mutant cells. For this reason we decided to transform the vps52Δ strain with YFP-Aut7 and either Cvt19-CFP or CFP-Apel1. Analysis of Aut7 provides an independent way to mark the site of vesicle formation (24, 71, 72). As predicted, in wild type cells grown in rich medium Cvt19 and prApel1 were both in the same structure as Aut7 (Fig. 7C) (25, 38), whereas in the vps52Δ strain
those two proteins no longer co-localized with Aut7 in 40–50% of the cells examined (Fig. 7C). Nitrogen starvation conditions reversed the defect and resulted in localization of the prApe1-Cvt19 complex to the correct destination (Fig. 7C).

Because Cvt9 mediates the correct targeting of the prApe1-Cvt19 complex to the site of Cvt vesicle formation (38), we decided to investigate the localization of this protein relative to Cvt19 and the PAS. Wild type and vps52Δ cells were trans-
formed with Cvt19-CFP and YFP-Cvt9 or with YFP-Aut7 and CFP-Cvt9. As expected, in wild type cells grown either in rich medium or starved for nitrogen, Cvt19 and Aut7 were both in the same location as Cvt9, e.g. the PAS (Fig. 7D) (25, 38). In contrast, in the absence of VPS52, Cvt9 maintained the co-localization with Cvt19 but not with Aut7 in 30% of the cell population (Fig. 7D). These observations suggest that at least one of the causes of the Cvt pathway block in vps52Δ cells is the inability to correctly target the prApe1-Cvt19-Cvt9 complex to the site of vesicle formation. Nitrogen deprivation was once again able to reverse the defect and direct the prApe1-Cvt19-Cvt9 complex to the Aut7-containing structure (data not shown).

DISCUSSION

Vps51 Is a Subunit of the VFT Complex—The Vps fifty-three (VFT) complex and the rab GTPase Ypt6 are required for the tethering of early endosome-derived vesicles with late Golgi membranes (Fig. 8) (33, 45). Ric1 and Rgp1 form another complex that plays a crucial role during this recognition event. These two proteins localize to the late Golgi compartment where they recruit and catalyze nucleotide exchange on Ypt6 (40). Ypt6-GTP then binds directly to the VFT complex on the incoming vesicles (40). Because the VFT complex is associated with the vSNARE Tlg1, this tethering association brings Tlg1 in proximity with Tlg2, the tSNARE on the late Golgi membranes, leading to the assembly of the SNARE bundle, the core of the membrane fusion reaction (45).

The VFT complex is composed of three subunits: Vps52, Vps53, and Vps54 (40, 46). In a screen designed to find new deletion strains affecting the Cvt pathway, we identified Ykr020 (originally named Cvt22) as the fourth component of this complex and we named it Vps51. Vps51Δ, but also vps53Δ, vps54Δ, and vps53Δ, vps54Δ cells, have a defect in prApe1 maturation when grown in rich medium (Fig. 1A). We started to consider the possibility that Vps51 was interacting with the VFT complex when the results of a genome-wide approach for the identification of yeast protein complexes by mass spectrometry appeared indicating a putative interaction between Vps51 and Vps52 (44). We performed three different analyses that confirmed that Vps51 was participating in the same sorting step as the VFT complex. Vps51Δ cells missort a population of Prc1 to the periplasmic space, have a fragmented vacuole, and fail to reuse the vSNARE Snc1, phenotypes that are shared by the vps52Δ, vps53Δ, and vps54Δ strains (46, 47) (Figs. 1A and 4). The Prc1 and vacuolar morphology results are also corroborated by two different genome-wide studies where all nonessential genes necessary for Prc1 sorting and homotypic vacuole fusion were identified (65, 73). Sncl needs to be retrieved from the early endosome back to the late Golgi to be reused for exocytosis (37). Tethering/fusion partners of the VFT complex, Tlg1, Tlg2, Ypt6, Ric1, and Rgp1, are essential for this transport route (37, 40). In the present study we show that the VFT complex and Vps51 are also required for this recycling step (Fig. 4).

We obtained direct evidence that Vps51 is part of the VFT complex through an affinity isolation analysis. Vps51 tagged with protein A pulled down each of the other components of the VFT complex (Fig. 5A). Previous work has established that the ratio between each subunit is 1:1:1 (46). Vps51 did not pull down itself indicating that there is only one Vps51 subunit per complex (Fig. 5B). Thus, we concluded that the VFT complex is a heterotetramer with a 1:1:1:1 stoichiometry.

Vps51 is a small 164-amino acid protein that contains 2–3 putative coiled-coil regions. This protein has characteristics that differ from those of the other VFT complex components. For example, Vps52, Vps53, and Vps54 depend on each other to avoid rapid degradation, whereas Vps51 is not necessary for their stability and conversely, these three VFT components are not required for maintaining the appropriate cellular levels of Vps51 (46) (Fig. 5, C and D). The Vps52-Vps53-Vps54 trimer is still present in the absence of Vps51, which probably accounts for a residual activity of the VFT complex that would explain the less severe phenotypes observed in the vps51Δ strain (Figs. 2, A and C, and 4). Another peculiarity is that Vps52, Vps53, and Vps54 have clear mammalian homologues whereas Vps51 does not (33, 74). In a paper submitted in parallel with ours, Siniossoglou and Pelham (73) show that Vps51 is the VFT complex component that binds the N terminus of Tlg1. Because this region and that of the closest mammalian Tlg1 homologue, syntaxin 6, are not conserved, they hypothesize that the mammalian VFT complex possesses a divergent subunit.

The VFT Complex Is Specifically Required for Cvt Vesicle Biogenesis but Not for Autophagy and Pexophagy—Autophagy, pexophagy, and the Cvt pathway utilize a common machinery
and only a few factors specific for one route are known (1, 3, 4, 7) (Table II). In this article we demonstrate that the VFT complex is one of those. The Cvt pathway operates during vegetative growth conditions and it assures the delivery of prApe1 from the cytosol to the vacuole lumen (5, 6). Under the same conditions, the absence of the VFT complex causes a block in prApe1 transport and in its consequent processing (Fig. 1, A and C). Interestingly, the same cells are able to correctly carry out autophagy and pexophagy (Figs. 1A and 2, A, C, and E). There are some delays in the kinetic rates of these processes with vps51Δ cells being the most similar to the wild type (Fig. 2, C and E). The missorting of vacuolar proteases in VFT complex mutants is not total, a fraction of those proteins normally reach the vacuole where they are processed and activated because of the correct acidification of this organelle (46, 47) (Fig. 1B). For this reason a reduced hydrolytic activity of the vacuole is not a likely explanation for the slower kinetics of autophagy and pexophagy seen in the vps51Δ and VFT mutant strains. Rather, these differences may reflect a slower cellular metabolism. However, analysis of autophagosomes by electron microscopy revealed that those structures have a reduced size in vps51Δ and vps52A cells indicating that the membrane expansion process may be impaired (Fig. 3). This is an interesting finding because it suggests that a specific block in the Cvt pathway reduces autophagosome size indicating that this route may be used to transport membranes to the PAS during nitrogen starvation. Alternatively, the VFT components and Vps51 may play some direct role in membrane delivery for autophagosome expansion. Further studies will be needed to fully identify the source membranes for Cvt vesicles and autophagosomes.

Tlg2, Tlg1, and Vps45 are also essential for the formation of Cvt vesicles but not for autophagosome biogenesis (31) (Fig. 6, B and C). It has been shown that the VFT complex interacts directly with Tlg1 and indirectly with Tlg2 (45). Here we demonstrated that indeed, there is a unique VFT complex composed of four subunits that transiently bind Tlg1 (Fig. 6A). Taking these results together, we can conclude that the VFT complex, Tlg2, and by extension Tlg1 and Vps45, are mediating a transport step essential for the formation/completion of Cvt vesicles.

The absence of the VFT complex results in a block of prApe1 import and the failure to deliver membranes that are required for Cvt vesicle assembly and will help to connect together all the specific proteins required for Cvt vesicle assembly and will help to elucidate in better detail the mechanism that leads to the formation of the PAS. Autophagy, however, employs factors not required for the Cvt pathway to supply the PAS with membranes suggesting that there are fundamental differences between the mechanism of formation of Cvt vesicles and autophagosomes. The comparison between the trafficking requirements for PAS formation during vegetative growth with those during starvation will improve our understanding of the reorganization of these trafficking routes in different environmental situations.

Cvt9 is a specific factor required for the Cvt pathway and pexophagy but not for autophagy (16) (Table II). Because Cvt9 is mislocalized in VFT complex mutants, one would expect that pexophagy would be impaired in the same cells. An interesting observation is that nitrogen deprivation triggers the correct targeting of Cvt9 to the PAS in the VFT mutants, furnishing an explanation as to why the prApe1 block is rapidly bypassed during autophagy (Fig. 7B). As mentioned, Cvt9 itself is not required for autophagy, but its proper re-localization may reflect the correct positioning of an associated factor that is essential for this pathway (16) (Table II). Nitrogen starvation conditions are also employed to induce peroxisome degradation (see “Experimental Procedures”). Thus, under those circumstances, Cvt9 is probably correctly localized so that this catalytic process proceeds normally (Fig. 2E). That leads us to conclude that prApe1-Cvt19 and pexisomes need a common element, Cvt9, to be targeted to the PAS, but those two pathways have different trafficking requirements with pexophagy probably using the same membrane source as autophagy.

The great specificity in vesicular traffic is achieved by the partnership between SNAREs and tethers and different factors (33, 34). These proteins can be involved in one or several different fusion events, but the combination between them creates a specific assortment utilized only for a unique vesicular trafficking step. For this reason, Tlg2, Tlg1, and the VFT complex are probably participating in one or more retrieval steps back to the Golgi (Fig. 8). Two hypotheses can explain the role of this fusion machinery in Cvt vesicle assembly. The first possibility is that there is a retrograde transport route from the PAS for specific proteins. One possible cargo molecule of this recycling pathway is App9. This transmembrane protein is localized to the PAS but it is not found on complete autophagosomes, indicating that it is retrieved prior to the fusion of those vesicles with the vacuole (24, 25, 77). The second hypothesis is that in the absence of this fusion machinery, the proper homeostasis of either the early endosome or Golgi complex is severely compromised, and that has an indirect effect on Cvt vesicle completion by altering the sorting of specific transmembrane proteins. Tlg2 also localizes to Cvt vesicles (31). For this reason, at the moment we cannot exclude that this tSNARE is playing additional roles in the homotypic fusion that leads to Cvt vesicle completion or in the transport of intermediate vesicles to the PAS that are required for vesicle vesicle fusion.

Future work will help to connect together all the specific proteins required for Cvt vesicle assembly and will help to elucidate in better detail the mechanism that leads to the formation of the PAS. Autophagy, however, employs factors not required for the Cvt pathway to supply the PAS with membranes suggesting that there are fundamental differences between the mechanism of formation of Cvt vesicles and autophagosomes. The comparison between the trafficking requirements for PAS formation during vegetative growth with those during starvation will improve our understanding of the reorganization of these trafficking routes in different environmental situations.

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