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Apg13p and Vac8p Are Part of a Complex of Phosphoproteins That Are Required for Cytoplasm to Vacuole Targeting*

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We have been studying protein components that function in the cytoplasm to vacuole targeting (Cvt) pathway and the overlapping process of macroautophagy. The Vac8 and Apg13 proteins are required for the import of aminopeptidase I (API) through the Cvt pathway. We have identified a protein-protein interaction between Vac8p and Apg13p by both two-hybrid and co-immunoprecipitation analysis. Subcellular fractionation of API indicates that Vac8p and Apg13p are involved in the vesicle formation step of the Cvt pathway. Kinetic analysis of the Cvt pathway and autophagy indicates that, although Vac8p is essential for Cvt transport, it is less important for autophagy. In vivo phosphorylation experiments demonstrate that both Vac8p and Apg13p are phosphorylated proteins, and Apg13p phosphorylation is regulated by changing nutrient conditions. Although Apg13p interacts with the serine/threonine kinase Apg1p, this protein is not required for phosphorylation of either Vac8p or Apg13p. Subcellular fractionation experiments indicate that Apg13p and a fraction of Apg1p are membrane-associated. Vac8p and Apg13p may be a part of a larger protein complex that includes Apg1p and additional interacting proteins. Together, these components may form a protein complex that regulates the conversion between Cvt transport and autophagy in response to changing nutrient conditions.

The majority of intracellular degradation is carried out in the lysosome/vacuole of eukaryotic cells. In order to compartmentalize these reactions, substrates as well as degradative enzymes must be faithfully delivered to this organelle. In Saccharomyces cerevisiae, proteins are known to be delivered to the vacuole for degradation by several different pathways (1). For example, cell surface proteins are transported by endocytosis; the cytosolic enzyme fructose-1,6-bisphosphatase is targeted to the vacuole for degradation by several different pathways (1). However, it is worth noting that under certain conditions macroautophagy may be a selective process. For example, peroxisomes can be selectively degraded by a macroautophagic process in response to changing nutrient conditions (reviewed in Ref. 9).

1 The abbreviations used are: PVC, prevacuolar compartment; ALP, alkaline phosphatase; API, aminopeptidase I; Arm, armadillo repeat; Cvt, Cytoplasm to vacuole targeting; PGK, phosphoglycerate kinase; PrA, protease A; SD-N, synthetic minimal medium lacking nitrogen; SMD, synthetic minimal medium containing nitrogen; NPM, low phosphate medium; prAPI, precursor aminopeptidase I; kb, kilobase pair(s); GFP, green fluorescent protein; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid.

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Apg13p and Vac8p Form a Protein Complex

Biochemical and morphological studies confirm that the basic properties of targeting by the Cvt pathway are similar to those of macroautophagy (3, 10). Both pathways involve formation of double membrane vesicles that engulf cytosolic cargo and deliver it to the vacuole by fusion of the outer membrane with the vacuole surface. When cells are growing in rich media, the primary mode of prAIP delivery is via Cvt vesicles (3). These vesicles are approximately 150 nm in diameter, relatively rare, and contain concentrated cargo that appears different than bulk cytosol. In contrast, in starvation conditions, the majority of prAIP appears to be targeted to the vacuole by autophagosomes. Compared with Cvt vesicles, autophagosomes are larger (approximately 300–900 nm), more abundant, and appear to contain bulk cytosolic components in addition to Cvt complexes. The mechanisms by which environmental changes are transduced to signal alterations in vesicle morphology and selectivity are not known. Recent cloning of the genes that complement mutants defective in the autophagy and Cvt pathways indicates that the majority of the molecular components involved in these pathways are shared (reviewed in Ref. 9). However, several mutants have been identified that appear to be defective primarily in one or the other of these pathways. These include aut4 (6), apg17,3, cvt3 (5), cvt9,5, tlg2 (11), and vac8 (12).

The vac8 mutant was identified through a screen for strains defective in vacuole inheritance (13). In addition to a role in the migration of vacuoles from the mother to the bud during cell division, Vac8p is required for Cvt transport. Analysis of vac8 mutants by a vesicle test that monitors the accumulation of subvacuolar vesicles under starvation conditions, however, provided preliminary evidence that this protein is not necessary for autophagy (12). The majority of Vac8p consists of 11 armadillo repeats. These domains are contained within the Drosophila armadillo protein and the mammalian homologues β-catenin and plakoglobin. The armadillo domains in β-catenin and plakoglobin serve to link regions of the plasma membrane to actin, and Vac8p co-sediments with actin filaments in vitro (12). Localization experiments indicate that Vac8p is found primarily on the vacuole membrane (12, 14, 15). In addition, Vac8p is both myristoylated and palmitoylated, and mutational analysis indicates that acylation is required both for vacuole localization of Vac8p and for vacuole inheritance. Interestingly, vac8 mutants defective in acylation are not defective in Cvt transport, suggesting that different cellular pools of Vac8p are utilized for inheritance and Cvt transport (12).

To identify proteins that interact with Vac8p, we have performed a two-hybrid screen using this protein as bait. We identified Apg13p as a Vac8p-interacting protein. Apg13p is predicted to be an 83-kDa protein, with no significant homology to other known proteins (16). Overexpression of APG1 suppresses the autophagy defect in the apg13–1 mutant, suggesting that these two proteins also interact (16). Mutants in APG13 are defective in both the Cvt and autophagy pathways (5, 16). We propose that these proteins are part of a large protein complex that functions in the vesicle formation step that is required for sequestration of cargo during import from the cytoplasm into the vacuole.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The *S. cerevisiae* strains used in this study are listed in Table I.

Yeast strains were grown in the indicated media: synthetic minimal medium (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed), YPD (1% yeast extract, 2% peptone, 2% glucose), synthetic minimal medium containing 2% glucose without ammonium sulfate or amino acids (SD-N), and low phosphate medium (NPM; 2% glucose, 0.5% casamino acids, 1% complete amino acid mix, 1% proline, 2 mM NaCl, 4 mM MgCl2). The Strain and Media—The *S. cerevisiae* strains used in this study 25841
ΔArm4–7); and pYW51, Arm1–3 (12).

**APG13** was amplified from strain SEY6210 genomic DNA into two overlapping fragments by two PCR reactions. For reaction one, the sense primer was 5′-CGGCGTCAGCAGCAGGTAAGGGAACGCTTTGCGGAGCG-3′ and the antisense primer was 5′-GGTGCTGCGTGTAGCTGCACTGATGGCTG-3′. For reaction two, the sense primer was 5′-GGTGTTACTGCTGGTAGTGTCG-3′, which adds a *SacI* site, and 5′-TAAAATAAA- AAGCTTTTTTGATTCTACTTCATGCGCTGG-3′, which adds a *HindIII* site. The amplified fragment was inserted into the *SacI* and *HindIII* sites of pTrcHisB to generate pTrcHisAPG13.

**Construction of the VAC8 Two-hybrid Bait Plasmid**—The VAC8 coding region was PCR-amplified with the primers V22 (5′-CATGGCACTGTGGGCTTACATGCTGGT-3′), which adds a *NcoI* site and V23 (5′-AACGCTTGACTAATGAAAATAAGGTATATCTCTCTTT-3′), which adds a *SalI* site. *NcoI*-SalI-digested VAC8 was cloned into pNossI-digested pAS2 (20) to create the VAC8 two-hybrid bait plasmid pYW31. The same strategy was used to clone the mutated *vac8* gene from pLYT4, pLYT5, pLYT6, pYW22, and pYW51 into the two-hybrid vector.

**Two-hybrid Library Screen**—The bait plasmid pYW31 was transformed into yeast strain JPH9-4A (21) and checked for activation of the reporter genes. The resulting strain JPH9-4A + pYW31 was transformed with the two-hybrid yeast library pool Y2H-H-C1 (21). Approximately 280,000 transformants were screened. The transformants were plated onto SC-His-Leu-Trp + 3 mM 3-amino-1,2,4-triazole and incubated at 24 °C for 14 days. These plates were replica plated onto SC-Ade-Leu-Trp and incubated at 24 °C for 5 days. Colonies were picked and tested for β-galactosidase activity. Library plasmids were isolated from the yeast strain and retransformed into JPH9-4A + pYW31. Plasmids that showed positive responses after retransformation were sequenced and identified by comparison with the Saccharomyces Genome Database. Two identical clones of *APG13* that coded for amino acids 567–738 were isolated.

**Construction of the APG13 Two-hybrid Prey Plasmids**—Plasmid pTrcHisAPG13 was digested with *PstI* and *HindIII*. The 2.2-kb *APG13* fragment was cloned into *PstI*-*HindIII*-cut pBlueScript SK to create pJN9. The following plasmids were generated by digesting pJN21 with the indicated enzymes, isolating DNA of the size noted, and ligating into pGAD-C (1–3) (7) that had been digested with the indicated enzymes to construct prey plasmids encoding the indicated number of amino acids of *APG13* (Table III), with p(JN23-APG13), *SalI* and partially digested with BamHI (2.9 kb), into *BamHI*-SalI sites of C2, full-length *APG13*; pJN23 (pAD1–520*APG13*), *BamHI* (1.6 kb), into *BamHI*-SalI sites of C2, amino acids 1–520; pJN24 (pAD1–279*APG13*), *BamHI*-SacI (0.8 kb), into *BamHI*-*SacI* sites of C2, amino acids 1–279; pJN25 (pAD280–520*APG13*), *NeiI*-SacI (0.8 kb), into *PstI*-*SacI* sites of C2, amino acids 280–520; pJN26 (pAD280–738*APG13*), *NeiI*-SacI (1.3 kb), into *PstI*-*SacI* sites of C2, amino acids 280–738; pJN27 (pAD521–692*APG13*), *BamHI*-SacI (0.7 kb), into *BamHI*-SacI sites of C3, amino acids 521–738; pJN28 (pAD521–692*APG13*), *BamHI*-ClaI (0.5 kb), into *BamHI*-ClaI sites of C3, amino acids 521–692; pJN29 (pAD693–738*APG13*), *ClaI*-SacI (0.2 kb), into *ClaI*-SacI sites of C1, amino acids 692–738.

Control plasmids for the two-hybrid analysis were pSE111 containing *SNF4* fused to the GALA activation domain in pMCT and pSE112 containing *SNF1* fused to the GALA binding domain in pAS1 (22).

**Subcellular Fractionation**—Yeast strains were grown to an *A* <sub>600</sub> = 0.8–1, and converted to spheroplasts as described (23). vac8Δ cells were grown in SMD, and *apg13Δ* cells were grown in YPD. Spheroplasts were washed in PS1000 (20 mM K-PIPES, pH 6.8, 1 mM sorbitol), and subjected to osmotic lysis (23) by pipetting up and down 10 times in PS200 with 5 mM MgCl₂ at a cell concentration of 20 *A* <sub>600</sub>/ml. The supernatant and pellet fractions were collected after centrifugation at 13,000 × g for 5 min at 4 °C.

**Pulse-Chase Labeling Analysis**—Pulse labeling experiments were as described (5). Yeast strains were grown in fresh medium at an *A* <sub>600</sub> = 0.8–1, pelleted, and then pulse-labeled at 30 °C in fresh SMD with 11 μCi/ml Expos<sup>35S</sup>35S-label. Chase reactions were initiated by addition of 10 μCi cytosine and 20 μCi methionine. Cells were then pelleted and resuspended at a concentration of 1 *A* <sub>600</sub>/ml in either SMD or SD-N for the duration of the chase reaction. API was immunoprecipitated as described (24).

**Electron Microscopy**—Indicated yeast strains were grown in YPD to an *A* <sub>600</sub> = 1, harvested, washed, incubated in SD-N for 4 h, and fixed with 1.5% KNO₃ as described (25).

**Phosphatase Labeling**—Cells were grown to an *A* <sub>600</sub> of 0.5–1 in NPM. 4 *A* <sub>600</sub> units of cells were harvested and labeled for 30 min at 30 °C in 100 μl of fresh NPM with 25 μCi of [32P]orthophosphate. Cells were recovered by centrifugation, and proteins were precipitated with 10% trichloroacetic acid and subjected to immunoprecipitation as described previously except that single precipitation reactions were performed.

**Co-immunoprecipitation Experiments**—B2168 cells harboring pYW10 and YEP351 (APG13) were grown in 100 ml of YPD, collected by centrifugation, washed with water, and resuspended in 50 ml of Z buffer (50 mM Tris-HCl, pH 7.5, 1 M sorbitol, 1% yeast extract, 2% peptone, 1% glucose) containing 0.5 mg/ml Zymolyase 100T. The suspension was incubated for 40 min at 30 °C with gentle shaking to generate spheroplasts. The resultant spheroplasts were washed once with Z buffer, resuspended in 50 ml of Z buffer, and divided into two 25-ml aliquots. Rapamycin (0.4 μg/ml, prepared in 90% ethanol, 10% Triton X-100) was added to one aliquot, and the spheroplasts were incubated for 1 h at 30 °C with gentle shaking. The spheroplasts were collected and washed with 50 mM Tris-HCl, pH 7.5, 1 M sorbitol, and lysed by suspending in buffer (phosphate-buffered saline, pH 7.4. 1 ml EDTA, 1 mM EGTA, 2 mM Na₂VO₃, 50 mM KF, 15 mM sodium pyrophosphate, 15 μM n-p-nitrophenylphosphate, 20 μg/ml leupeptin, 20 μg/ml benzamidine, 10 μg/ml pepstatin A, 40 μg/ml aprotinin, 1 mg phenylmethylsulfonyl fluoride, and 0.5% Tween 20). The cell lysate was further incubated on ice for 5 min, and spun down at 6,500 rpm for 10 min at 4 °C to remove cell debris. The resultant supernatant was collected and incubated with 20 μl of protein G-Sepharose (50% suspension) for 30 min at 4 °C with gentle agitation. The cell lysate was spun down, and the supernatant (two 50-μl aliquots) was incubated with or without 2 μl of anti-Vac8p serum for 1.5 h at 4 °C with gentle agitation. 20 μl of protein G-Sepharose (50% suspension) was added to the lysate/anti-serum mixture, followed by further incubation for 1.5 h at 4 °C with gentle agitation. The immune complex was washed three times with lysis buffer, and the resulting immunocomplex was subjected to immunoblotting using anti-Apg13p antibody (1:5,000 dilution) or anti-Vac8p antibody (1:5,000 dilution). For secondary antibody, horseradish peroxidase-conjugated anti-rabbit goat antibody (1:10,000) was used. Immunodetection was carried out with the ECL system.

**RESULTS**

**Vac8p and Apg13p Interact**—The vac8Δ mutant was identified in a screen for strains defective in vacuolar inheritance (13). Vac8p is unusual among those proteins involved in organelle segregation in that it was also found to be required for the import of the resident vacuolar hydrolase aminopeptidase I through the cytoplasm to vacuole targeting pathway. To gain further information on the role of Vac8p in the Cvt pathway, a two-hybrid screen was used to identify proteins that interact with Vac8p. The VAC8 gene was cloned into a two-hybrid vector containing a DNA encoding domain of his3 ade2 strain transformed with the resulting pBD-VAC8 plasmid and a plasmid library that had been cloned into the transcriptional activating domain vector. Transformants were screened for growth on His-free plates, and then growth on Ade-free plates and finally for β-galactosidase activity. Plasmids were recovered from cells that were positive by all three criteria and were subjected to DNA sequencing. One of the genes that was iden-
tified from the screen was found to contain part of the open reading frame corresponding to \textit{APG13} (Fig. 1A). The \textit{APG13} gene was identified as being required for macroautophagy (16) and was subsequently shown to be needed for import of prAPI (5).

To determine the domain of Apg13p that interacts with Vac8p, a series of deletions was constructed in the \textit{APG13} gene that was used as the prey plasmid for the two-hybrid interaction. \textit{APG13} constructs lacking the sequence for the C-terminal 46 amino acids showed a dramatically reduced interaction with a full-length \textit{VAC8} bait plasmid (Fig. 1B). Conversely, an \textit{APG13} prey construct encoding only the C-terminal 46 amino acids of Apg13p also was unable to promote interactions with Vac8p. These results suggest that the C terminus of Apg13p, including but possibly extending beyond the final 46 amino acids, is required for the interaction. However, additional data suggest that more than one domain of Apg13p may be involved in the interaction with Vac8p. Apg13p lacking amino acids 568 to 738 is able to partially complement the prAPI import defect of an \textit{apg13Δ} strain.\(^2\) The truncated Apg13p protein does not function as efficiently as full-length Apg13p, suggesting that the absence of the interaction domain causes a kinetic defect in prAPI import.

The bulk of the structure of Vac8p consists of 11 consecutive armadillo (Arm) repeats, each of 40–42 amino acids in length. These domains are thought to mediate protein-protein interactions (26). To determine if the interaction of Vac8p and Apg13p depends on the presence of Arm repeats within Vac8p, we tested the effect of deleting various Vac8p Arm domains by two-hybrid analysis. We found that, with the exception of Arm 1, all the Arm deletions examined were required for detection of the Vac8p-Apg13p interaction by the two-hybrid method (Fig. 1C). All of these Vac8p Arm deletion constructs produce stable proteins in yeast (data not shown). Next, we examined the effect of the Arm deletions on transport of prAPI. By immunoblot analysis, with the exception of the Arm 1 deletion, prAPI accumulated in cells where the only Vac8p present contained an Arm deletion (Fig. 1D). The fact that mutations that appear to prevent interaction of Vac8p and Apg13p also inhibit Cvt transport suggests that the Arm domains are important for Vac8p function. Furthermore, these results suggest that the interaction between Vac8p and Apg13p is needed for efficient import of prAPI through the Cvt pathway.

To verify that the native Vac8 and Apg13 proteins interact \textit{in vivo}, we performed a co-immunoprecipitation analysis. Protein extracts were prepared from yeast cells under native conditions and immunoprecipitated with antiserum to Vac8p. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and Apg13p was detected by immunoblot using anti-Apg13p.
antiserum as described under “Experimental Procedures.” When the immunoprecipitation was carried out in a strain overexpressing \textit{VAC8}, but with a deletion in the chromosomal \textit{APG13} locus, only \textit{Vac8p} was detected on the immunoblot, indicating that there were no background bands that migrated at the position of \textit{Apg13p} (Fig. 2, lane 1). Similarly, in the absence of antiserum to \textit{Vac8p}, neither protein was detected on the immunoblot, indicating that they did not bind nonspecifically to the protein G-Sepharose beads (Fig. 2, lanes 2, 4, and 6). In contrast, \textit{Apg13p} was found to co-immunoprecipitate with anti-Vac8p antiserum when both \textit{Vac8p} and \textit{Apg13p} were expressed from multicopy plasmids (Fig. 2, lane 3). This result confirms that \textit{Vac8p} and \textit{Apg13p} interact in vivo. The drug rapamycin mimics starvation conditions and induces autophagy by inhibiting the Tor kinase (27). \textit{Apg13p} and \textit{Vac8p} were found to interact in vivo following treatment with rapamycin (Fig. 2, lane 5). Similarly, \textit{Vac8p} and \textit{Apg13p} were found to interact under starvation conditions based on the two-hybrid analysis (data not shown). These results mean that \textit{Vac8p} and \textit{Apg13p} interact under both vegetative conditions where the Cvt pathway functions and starvation conditions that induce autophagy.

Both genetic and biochemical data indicate that \textit{Apg13p} interacts with another protein required for both the Cvt pathway and autophagy, the serine/threonine kinase \textit{Apg1p} (16, 28, 29). In addition, recent two-hybrid results showing that \textit{Apg13p} and \textit{Apg1p} interact corroborate these findings (29). The \textit{Vac8p}/\textit{Apg13p} interaction was not dependent on the presence of \textit{Apg1p} because similar two-hybrid results were obtained in an \textit{apg1\Delta} strain (Fig. 1A). These result suggest that \textit{Vac8p} and \textit{Apg13p} interact independent of \textit{Apg1p}.

\textbf{Strains with Mutations in \textit{VAC8} and \textit{APG13} Are Defective in the Membrane Enwrapping Step of the Cvt Pathway—}Biochemical localization of prAPI has been used to determine the step of the Cvt pathway that is blocked in various \textit{apg} and \textit{cvt} mutants (10). Mutants that are blocked in vesicle formation accumulate Cvt complexes, indicated by the appearance of prAPI in a low speed pellet fraction. Fractionation analyses demonstrate whether the Cvt complex is associated with membrane. Protease protection assays distinguish between prAPI trapped in a Cvt complex that is not fully surrounded by membrane and is therefore protease-accessible in permeabilized cells, and prAPI trapped in Cvt vesicles that appears protease-protected. Further biochemical fractionation of mutants that accumulate protease-protected prAPI demonstrates whether prAPI is contained within cytosolic vesicles (Cvt vesicles) or subvacuolar vesicles (Cvt bodies). By these analyses, \textit{Apg5p}, \textit{Apg7p}, \textit{Apg9p}, and \textit{Aut7p} have been shown to affect Cvt vesicle formation/completion (30–33); \textit{Vam3p}, \textit{Ypt7p}, and \textit{Vps18p} are required for Cvt vesicle fusion (10, 31, 34); and \textit{Cvt17p} is required for the breakdown of Cvt bodies within the vacuole (10).

We examined the state of prAPI to determine the step of the Cvt pathway that is blocked in the \textit{vac8\Delta} and \textit{apg13\Delta} mutants. Spheroplasts isolated from either \textit{vac8\Delta} or \textit{apg13\Delta} strains were subjected to differential osmotic lysis under conditions that have been shown to efficiently lyse the plasma membrane while preserving the integrity of most vacuoles (4, 23, 35). These permeabilized spheroplasts were then separated into pellet and supernant fractions by centrifugation at 5000 \times g. In both mutant strains, the majority of prAPI was found in the pellet fraction (Fig. 3, A and B). We examined the localization of the vacuolar hydrolase PrA and the cytosolic protein PGK in the same samples as controls for the fractionation. The majority of PGK was recovered in the supernatant fraction, while PrA was found in the pellet fraction, indicating that the fractionation was successful (Fig. 3, A and B).

To determine if the prAPI recovered in the pellet fraction was in fact associated with membrane components, we performed a flotation analysis using the pellet fraction as the starting material. The prAPI trapped in both the \textit{vac8\Delta} and \textit{apg13\Delta} strains floated through a Ficoll gradient in the absence, but not in the presence, of detergent, suggesting that this accumulation of prAPI is membrane-associated. It is possible that flotation of prAPI under these conditions reflects binding to lipid rather than a membrane bilayer. However, in an \textit{apg5\Delta} mutant we were able to verify through electron microscopy that flotation of prAPI corresponded with membrane association (30). When permeabilized cells from the \textit{vac8\Delta} and \textit{apg13\Delta} strains were examined by protease treatment, the majority of prAPI was accessible to added protease in both the presence
and absence of detergent (Fig. 3, A and B). Because the vacular hydrolase PrA is primarily recovered in the pellet fraction, intracellular compartments such as vacuoles (which are one of the most osmotically labile subcellular compartments) are intact in these samples. These results suggest that prAPI is localized in a membrane-associated Cvts complex, and that Vac8p and Apg13p are required for formation of the fully membrane enclosed Cvts vesicle.

Vac8p Is Not Essential for Autophagy—We have reported previously that autophagic bodies are detected in vac8Δ cells by light microscopy, and thus Vac8p appeared not to be required for autophagy (12). Because the majority of molecular components are shared between the Cvts and autophagy pathways, we further investigated the role of Vac8p in autophagy. Precursor API is transported by the Cvts pathway in nutrient-rich conditions and by the autophagy pathway in starvation conditions (3, 5). Accordingly, we examined the kinetics of prAPI maturation in different nutritional conditions as a biochemical method of examining the autophagy defect in the vac8Δ and apg13Δ strains. In wild type cells, prAPI is matured with a half-time of about 30 min in both SMD and SD-N media (Fig. 4A). In contrast, in an apg13Δ strain, no mature API is detected in either nutrient condition. In apg13Δ cells, API maturation is essentially completely blocked in YPD medium (data not shown). In contrast, some mature API is detected after a 90-min chase in SMD medium. When these cells are chased in SD-N, about 70% of API is mature at the 90-min chase point (Fig. 4B). In vac8Δ cells, prAPI delivery is negligible in nitrogen-containing medium, and restored to near wild type levels in starvation medium. These results suggest that prAPI can be efficiently transported by autophagy in vac8Δ cells during starvation, and that Vac8p has a more central role in the Cvts pathway than in the autophagy pathway. Furthermore, the defect in prAPI import in the apg13Δ strain can be partially overcome under starvation conditions.

To examine the kinetics of autophagy in these mutants directly, we utilized an alkaline phosphatase construct that was designed as a marker for the autophagy pathway (36). This protein, called Pho8Δ60p, consists of alkaline phosphatase in which the membrane-spanning region and the vacuole targeting information have been deleted (18, 37). The resulting cytosolic protein is only delivered to the vacuole when autophagy is induced. Pho8Δ60p still contains a site for cleavage of the C-terminal propeptide by the vacuole hydrolase proteinase B. Therefore, vacuolar delivery of Pho8Δ60p results in cleavage to a lower molecular weight species, and activation of enzyme activity. A pulse-chase experiment was performed to compare the kinetics of the autophagic uptake of Pho8Δ60p in wild type, vac8Δ, and apg13Δ cells (Fig. 4C). In wild type cells, maturation of Pho8Δ60p proceeds rapidly to about the 8-h chase point and then plateaus at about 18% delivery. In contrast, the apg13Δ strain matures only a very low level of Pho8Δ60p, consistent with its classification as an autophagy mutant. In vac8Δ cells, an intermediate level of Pho8Δ60p maturation was observed, suggesting that the autophagy pathway is active but is not as efficient as in wild type cells.

To confirm that the autophagy pathway is carried out in vac8Δ cells, we examined these cells directly by electron microscopy. Autophagy results in the delivery of an intact vesicle called an autophagic body to the vacuole lumen. In cells deficient in PrA, these vesicles cannot be broken down and accumulate in starvation conditions (38). We examined the effect of the vac8 and apg13 deletions on vesicle accumulation in cells in which PEP4, the gene encoding PrA, had been deleted. In pep4Δ cells after incubation in starvation conditions for 4 h, numerous autophagic bodies were detected in the vacuole (Fig. 5A). In the apg13Δ pep4Δ double mutant, the vacuoles appeared empty as expected, confirming a block in autophagy resulting from the apg13 mutation (Fig. 5B). In contrast, in the vac8Δ pep4Δ double mutant, autophagic bodies appeared to be as abundant as in the pep4Δ cells (Fig. 5, compare A to C). Cells lacking Vac8p have been shown to have fragmented vacuoles (12). This was apparent in many of our images, which contained multilobed or vesiculated vacuoles containing subvacuolar vesicles (data not shown). These results confirm that autophagy proceeds in the vac8Δ strain.

Vac8p Is a Phosphoprotein—Apg13p is a phosphoprotein that interacts with Apg1p. Although Apg1p is both a phosphoprotein and a protein kinase (28), the phosphorylation of Apg13p does not appear to depend on Apg1p (Fig. 6C). To determine if Vac8p is also a phosphoprotein, cells were metabolically labeled for 10 min in nitrogen-containing medium, washed, and chased for the indicated times (min). API was recovered by immunoprecipitation. Note that the band running slightly below the position of mature API in the apg1Δ strain is a background band. The positions of precursor (pr) and mature (m) API are indicated. B, quantitation of the 90-min time point of the samples in A are depicted. The black bars represent the SMD chase, and the white bars the SD-N chase. The amount of mature API in the apg1Δ strain in either condition was less than 1%. C, kinetics of Pho8Δ60p delivery.

Cells (wild type, TN121; vac8Δ, D3Y103; apg13Δ, D3Y104; apg13Δ, NNY20) were metabolically labeled for 10 min as described under “Experimental Procedures.” After labeling, the cells were resuspended and resuspended in either SMD or SD-N and chased for the indicated times (min). API was recovered by immunoprecipitation. Note that the band running slightly below the position of mature API in the apg1Δ strain is a background band. The positions of precursor (pr) and mature (m) API are indicated. B, quantitation of the 90-min time point of the samples in A are depicted. The black bars represent the SMD chase, and the white bars the SD-N chase. The amount of mature API in the apg1Δ strain in either condition was less than 1%. C, kinetics of Pho8Δ60p delivery.

Cells (wild type, TN121; vac8Δ, D3Y103; apg13Δ, D3Y104; apg13Δ, NNY20) were metabolically labeled for 10 min in nitrogen-containing medium, washed, and chased for the indicated times in SD-N. Pho8Δ60p was recovered by immunoprecipitation with anti-ALP antiserum, resolved by SDS-polyacrylamide gel electrophoresis, and quantified using a STORM PhosphoImager (Molecular Dynamics, Sunnyvale, CA).
bolically labeled with $[^{33}P]$orthophosphate, and Vac8p was recovered by immunoprecipitation. Labeled Vac8p was recovered from cell lysates (Fig. 6A), indicating that it is a phosphoprotein. In addition, the level of Vac8p phosphorylation was not changed in an *apg1Δ* strain, suggesting that Apg1p is not the kinase responsible for Vac8p phosphorylation. Apg1p also interacts with Cvt9p. 3 Like Vac8p, Cvt9p appears to play a more predominant role in Cvt transport than in the autophagy pathway. To determine if Cvt9p is phosphorylated, cells were again subjected to metabolic labeling with $[^{33}P]$orthophosphate, and Cvt9p was recovered by immunoprecipitation. As is the case for Apg13p and Vac8p, Cvt9p is a phosphoprotein that does not appear to depend on Apg1p for phosphorylation, despite the fact that Apg1p and Cvt9p form a complex.

The level of Apg13p phosphorylation is dependent on nutritional conditions. 2 The protein is hyperphosphorylated in rich media and undergoes partial dephosphorylation in starvation conditions. Because phosphate starvation conditions were required to get incorporation of labeled phosphate for these experiments, it was not possible to compare the level of phosphorylation in starvation and non-starvation conditions by the labeling method. However, Apg13p hyperphosphorylation results in a shift in relative mobility that we could detect by SDS-polyacrylamide gel electrophoresis and immunoblot. For these experiments, cells were grown in rich medium and then shifted to SD-N at time 0. Upon shifting to nitrogen starvation conditions, Apg13p was rapidly dephosphorylated as indicated by the presence of a lower molecular weight immunoreactive band (Fig. 6B). In contrast, the migration of Apg1p is not affected by the change in available nitrogen. Because migration of Apg1p is not affected by nutrient conditions, it is not possible to use this method to determine if the level of Apg1p phosphorylation is nitrogen-dependent.

We also examined other mutant strains for effects on regulation of Apg13p phosphorylation. Although most of the mutant strains we examined including *cvt9Δ* and *apg1Δ* did not alter the starvation induced dephosphorylation of Apg13p, we did detect a change in the phosphorylation pattern of Apg13p in *vac8Δ* cells (Fig. 6C). In wild type cells, the majority of Apg13p was present in a high molecular weight form when the cells were grown in rich media. In contrast, in *vac8Δ* cells, only about 50% of Apg13p existed in the hyperphosphorylated state in these conditions. These results suggest that hyperphosphorylation of Apg13p in rich media is impaired in the absence of Vac8p.

Apg13p and Apg1p Are Membrane-associated—Vac8p is associated with the vacuole membrane (12, 14, 15). To determine if Apg13p and Apg1p are also membrane-associated proteins, subcellular fractionation experiments were performed. Both Apg13p and Apg1p are expressed at low levels in the cell and are labile in cell lysates. In order to detect these proteins in subcellular fractionation experiments, overexpression of these proteins was necessary, and even with the addition of protease inhibitors, it was not possible to perform prolonged fractionation procedures. Spheroplasts prepared from cells overexpressing either Apg13p or Apg1p were lysed osmotically and separated into supernatant and pellet fractions. After centrifugation at 13,000 $\times$ g, Apg13p was recovered completely in the pellet fraction (Fig. 7A). As expected, Vac8p was also recovered in the pellet fraction, while the cytosolic marker PGK was recovered in the supernatant fraction, confirming that membrane and soluble proteins are effectively separated in this
from that of the native Apg13 protein, i.e. fusions to Apg13p may in some cases interfere with its ability to interact with other proteins. Both native Vac8p and Apg13p are membrane-associated. For Apg13p, membrane binding is independent of Vac8p (Fig. 7). The ability to bind the target membrane may allow the truncated Apg13p to interact inefficiently with Vac8p due to a close proximity even in the absence of the C-terminal Vac8p-binding domain. Alternatively, it is possible that the truncated Apg13p does not bind Vac8p but is able to function in the Cvt pathway through its interaction with other components. Subcellular fractionation experiments demonstrate that prAPI, the only known cargo of the Cvt pathway, is trapped in a membrane-bound, protease-accessible state in both the vac8Δ and apg13Δ strains, suggesting that both proteins are required for the formation/completion of Cvt vesicles (Fig. 3).

Vac8p has several cellular roles and appears to be an abundant protein compared with other identified autophagy components. Other Arm proteins such as β-catenin/armadillo also exert multiple functions through interaction with different binding partners. The crystal structure of β-catenin has been solved (39). The Arm repeats form a single rod-like structure with a hydrophobic core. Each Arm domain folds into three α-helical segments connected by short spacers. These helices are then packed into a superhelical structure with a positively charged groove that is thought to form a surface for interaction with other proteins.

Vac8p is primarily located on the vacuole and requires acylation for both correct localization and its role in vacuole inheritance (12). The fact that Vac8p acylation is not required for Cvt transport suggests that a different subset of the Vac8p population may be utilized for Cvt transport (12). This population of Vac8p is likely to be in a complex with Apg13p and bound to a membrane fraction that may or may not be the vacuole. In addition, other components such as Cvt9p that may interact, perhaps indirectly, with Vac8p appear to be located in proximity to the vacuole. This can also be observed that Vac8p associates with the Cvt vesicle and is required for a fusion step that results in vesicle completion.

Vac8p and Apg13p are part of the same protein complex that includes Apg1p and Cvt9p. However, the respective mutant strains display somewhat different phenotypes (Figs. 4 and 5). The apg1Δ strain is completely blocked in autophagy and prAPI import in rich media or starvation conditions. In synthetic minimal medium containing nitrogen, apg1Δ cells slowly mature prAPI and the level of import is increased in medium lacking nitrogen. However, the apg1Δ strain is defective for autophagy, as assessed both morphologically and by maturation of Pho8Δ60p. The vac8Δ strain is completely blocked for prAPI import in YPD or SMD. In contrast, in SD-N this strain shows import kinetics that only slightly lag behind those of the wild type strain. Similarly, the vac8Δ cells appear to carry out autophagy in SD-N medium although at a substantially reduced rate.

One explanation for these results is that Vac8p is specific to the Cvt pathway. Accordingly, the induction of autophagy allows efficient import of prAPI in the vac8Δ strain. The partial defect in autophagy seen in the vac8Δ strain could be an indirect effect of the null mutation or might be due to an alteration in the state of the interacting protein Apg13p. It is also possible that Vac8p plays a role in autophagosome formation but is not essential for this process so that vac8Δ cells display only a kinetic defect in autophagy. The apg1Δ cells may carry out autophagy at a level that is too low to detect based on delivery of Pho8Δ60p or electron microscopy. However, this level of autophagy may be sufficient to account for the selective import experiment. Apg13p is predicted to be a soluble protein based on sequence analysis. To determine if its membrane localization is mediated through Vac8p, Apg13p fractionation was also examined in vac8Δ cells. As in wild type cells, Apg13p was recovered in the pellet fraction in vac8Δ cells, indicating it does not depend on its interaction with Vac8p for membrane localization (Fig. 7B). In contrast to these results, a GFP-Apg13p fusion protein displayed a diffuse cytosolic localization (data not shown). Although this fusion protein complemented the prAPI accumulation phenotype of the apg13Δ strain, it is possible that it has an altered subcellular distribution due to steric interference by GFP.

Apg1p is also predicted to be a soluble protein based on its deduced amino acid sequence. However, when the subcellular distribution was examined, about 50% of Apg1p was recovered in at 13,000 g in the 13,000 g pellet fraction (Fig. 7C). As before, Vac8p and PGK were recovered primarily in the pellet and supernatant fractions, respectively. When the 13,000 g supernatant fraction from these experiments was subjected to centrifugation at 100,000 g, Apg1p remained in the supernatant fraction (data not shown). Because some Apg1p was degraded in this longer experiment, we cannot rule out the possibility that a fraction of Apg1p may be in the 100,000 g pellet fraction. However, it appears that the bulk of Apg1p is distributed between a 13,000 g pellet fraction and a soluble pool.

Because both Apg13p and Apg1p are required for autophagy, and these proteins are thought to form a more stable complex in starvation conditions, localization of these proteins was also examined after incubation for 4 h in SD-N medium. This treatment did not affect the localization of either protein. Apg13p was still recovered in the 13,000 g pellet fraction, and Apg1p was distributed between the supernatant and pellet fractions (data not shown).

**DISCUSSION**

Vac8p is required for vacuole inheritance and Cvt transport. Vac8p contains 11 armadillo (Arm) repeats. This structural domain has been found in a number of proteins with diverse functions and may serve as a scaffold for protein-protein interactions (26). Two-hybrid analysis and co-immunoprecipitation experiments demonstrate that Vac8p and Apg13p physically interact with each other, and this interaction appears to be mediated through Arm domains in Vac8p (Figs. 1 and 2). A C-terminal domain of Apg13p in the context of a two-hybrid protein was shown to be required for interaction with a corresponding Vac8p two-hybrid construct. Surprisingly, deletion of this domain in native Apg13p still allowed the truncated protein to partially complement the Cvt pathway defect in an apg13Δ strain. One explanation may be the presence of additional sites in Apg13p that allow interaction with Vac8p. These sites may not be functional in the context of the two-hybrid protein. A similar situation may be seen with the GFP-Apg13p hybrid protein that displays a subcellular distribution distinct...
of prAPI observed in our experiments. An alternative explanation is afforded by the observation that the apg13 mutant is leaky for the Cvt pathway in nitrogen-containing SMD medium. This mutant may carry out transport of prAPI through the formation of Cvt vesicles during both vegetative and starvation conditions. Because Cvt vesicles are of comparatively low abundance, they may not have been detected by our electron microscopy analysis. Finally, it is possible that Applg13p is required for both pathways but like Vac8p it can be bypassed. For example, overexpression of Applp suppresses the apg13–1 mutation (16).

Vac8p and Applp, the Applp-interacting protein Applp, and the Applp-interacting protein Cvt9p are phosphorylated in vivo (Fig. 6). Applp and Cvt9p are thought to interact directly with Applp, which is proposed to be a protein kinase. However, these proteins are phosphorylated in apg13Δ cells, suggesting that another kinase is involved. Applp is hyperphosphorylated in rich media conditions; when cells are shifted to a poor nitrogen source, it is rapidly dephosphorylated (Fig. 6B). This nutrient-dependent change in the level of Applp phosphorylation could signal the change from transport via the Cvt pathway to the macroautophagy pathway. Hyperphosphorylation of Applp is reduced in vac1Δ cells (Fig. 6C), suggesting that the Vac8p/Applp complex may be a better substrate for phosphorylation than Applp alone and that Vac8p may help regulate Applp hyperphosphorylation.

Both Applp and Applp are predicted to be soluble proteins based on sequence analysis. In addition, a fusion protein where the first 32 amino acids of Applp/Aut3p were replaced with GFP appeared to be cytosolic (40). However, because this protein did not complement the autophagy defect in apg13Δ cells, it may not represent the localization pattern of authentic Applp. Similarly, a GFP-Applp fusion protein displayed a cytosolic fluorescence pattern, although in this case the protein was functional in the Cvt pathway (data not shown). Our fractionation experiments examining the native proteins expressed from multicopy plasmids indicate that Applp is localized in the P13 fraction, while Applp is distributed between a soluble fraction and the P13 fraction (Fig. 7). Both Applp and Applp are unstable during subcellular fractionation procedures and are present at low levels in the cell. To facilitate detection, these proteins were overexpressed. In the case of Applp, this may have resulted in the saturation of an Applp membrane binding site(s), resulting in the observed soluble pool. Alternatively, Applp may cycle between pools both on and off the membrane. The localization of Applp and Applp to the P13 fraction is consistent with their localization to either the vacuole, a forming Cvt vesicle, or possibly the PVC.

In our current model (Fig. 8), Applp and Vac8p may be part of a larger protein complex that includes Applp, and Cvt9p, and may include additional proteins; recently, Applp was found to interact with Applp,2 Applp, Vac8p, Applp (28), and Cvt9p are all phosphorylated proteins, and the level of Applp phosphorylation is regulated by the availability of nitrogen.

Applp and Vac8p are found in a protein complex in rich media as well as in the presence of rapamycin (Fig. 2). Applp and Applp interact more tightly in starvation conditions when Applp is in the lower phosphorylation state.2 The change in the level of Applp phosphorylation may allow additional Applp and associated proteins such as Cvt9p to be recruited to the Applp/Vac8p complex. This event may, in turn, be involved in switching from the Cvt pathway to autophagy.

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