Cvt9/Gsa9 Functions in Sequestering Selective Cytosolic Cargo Destined for the Vacuole

John Kim,* Yoshiaki Kamada,‡ Per E. Stromhaug,§ Ju Guan,* Ann Hefner-Gravink,* Misuzu Baba,¶ Sidney V. Scott,** Yoshinori Ohsumi,‡ William A. Dunn, Jr.,i and Daniel J. Klionsky*

*Department of Biology, University of Michigan, Ann Arbor, Michigan 48109; ‡Department of Cell Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan; §Institute for Cancer Research, Department of Cell Biology, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway; iDepartment of Anatomy and Cell Biology, University of Florida College of Medicine, Gainesville, Florida 32610; ¶Department of Chemical and Biological Sciences, Faculty of Science, Japan Women’s University, Tokyo 112, Japan; and **Section of Microbiology, University of California at Davis, Davis, California 95616

Abstract. Three overlapping pathways mediate the transport of cytoplasmic material to the vacuole in Saccharomyces cerevisiae. The cytoplasm to vacuole targeting (Cvt) pathway transports the vacuolar hydrolase, aminopeptidase I (API), whereas pexophagy mediates the delivery of excess peroxisomes for degradation. Both the Cvt and pexophagy pathways are selective processes that specifically recognize their cargo. In contrast, macroautophagy nonselectively transports bulk cytosol to the vacuole for recycling. Most of the import machinery characterized thus far is required for all three modes of transport. However, unique features of each pathway dictate the requirement for additional components that differentiate these pathways from one another, including at the step of specific cargo selection.

We have identified Cvt9 and its Pichia pastoris counterpart Gsa9. In S. cerevisiae, Cvt9 is required for the selective delivery of precursor API (prAPI) to the vacuole by the Cvt pathway and the targeted degradation of peroxisomes by pexophagy. In P. pastoris, Gsa9 is required for glucose-induced pexophagy. Significantly, neither Cvt9 nor Gsa9 is required for starvation-induced nonselective transport of bulk cytoplasmic cargo by macroautophagy. The deletion of CVT9 destabilizes the binding of prAPI to the membrane and analysis of a cvt9 temperature-sensitive mutant supports a direct role of Cvt9 in transport vesicle formation. Cvt9 oligomers peripherally associate with a novel, perivacuolar membrane compartment and interact with Apg1, a Ser/Thr kinase essential for both the Cvt pathway and autophagy. In P. pastoris Gsa9 is recruited to concentrated regions on the vacuole membrane that contact peroxisomes in the process of being engulfed by pexophagy. These biochemical and morphological results demonstrate that Cvt9 and the P. pastoris homologue Gsa9 may function at the step of selective cargo sequestration.

Key words: autophagy • degradation • lysosome • peroxisome • vacuole

Introduction

General cell function and homeostasis require the regulated counterbalancing of protein synthesis and organelle biogenesis with protein breakdown and the selective degradation of organelles. The macroautophagy pathway directs the lysosome-mediated breakdown of bulk cytoplasm, including proteins, nucleic acids, lipids, and organelles and serves as the principal cellular degradative process (for review see Klionsky and Ohsumi, 1999; Kim and Klionsky, 2000). The general mechanism of autophagy has been conserved from yeast to mammals. In yeast, starvation conditions mobilize the autophagic machinery to deliver cytoplasmic constituents to the vacuole, where substrate digestion and recycling mechanisms ensure cell survival. In mammals, macroautophagy has also been implicated to play a role in cellular remodeling during development, differentiation, and aging (Mortimore et al., 1996; Vittorini et al., 1999). In addition, recent findings that a dysfunctional autophagy pathway may contribute to the metastasis of certain types of cancers further underscore the relevance of autophagic degradation processes in humans (Liang et al., 1999; for review see Klionsky and Emr, 2000).

Biochemical and microscopy studies have outlined the basic steps of macroautophagy in Saccharomyces cerevisiae (for review see Klionsky and Ohsumi, 1999). Upon induction, double-membrane vesicles, termed autophagosomes, capture bulk cytoplasm before being targeted to the vacuole.
Fusion of the autophagosome with the vacuole membrane releases a single-membrane vesicle, the autophagic body, into the vacuolar lumen. Subsequent breakdown of the autophagic body releases its cytoplasmic contents for digestion and recycling.

Insights into the molecular details of macroautophagy have been greatly facilitated through molecular genetic studies in *S. cerevisiae* (Tsukada and Ohsumi, 1993; Thumm et al., 1994; for review see Kim and Klionsky, 2000). The cloning of the genes that complement these yeast macroautophagy mutants, *apg* and *aut*, has subsequently led to the identification of mammalian homologues of key macroautophagy components, thus supporting the conserved nature of this pathway across eukaryotic kingdoms. The identified machinery includes a group of components required for transducing the signal for autophagy induction to downstream effectors. Chief among these is the phosphatidylinositol 3-kinase homologue, Tor. Activation of Tor represses autophagy, whereas its inhibition by nutrient starvation conditions or with the immunosuppressant drug rapamycin triggers the pathway (Noda and Ohsumi, 1998). The activity of Tor also affects the phosphorylation state of Apg13 and the downstream kinase activity of Apg1 (Kamada et al., 2000). Tor-dependent hyperphosphorylation of Apg13 lessens the affinity of Apg13 for the Apg1 kinase and results in reduced kinase activity as well as the inhibition of autophagy. Conversely, Tor repression leads to decreased phosphorylation of Apg13, an increase in the interaction between Apg13 and Apg1, and the induction of autophagy.

The formation and completion of autophagosomes requires the function of a novel Apg protein conjugation system which results in the covalent linkage of Apg12 to Apg5 (Kametaka et al., 1996; Mizushima et al., 1998; George et al., 2000). The intermediate steps leading to Apg12–Apg5 conjugation involve Apg7, an E1 ubiquitin activating enzyme homologue, and Apg10, which functions as a protein conjugating enzyme (Mizushima et al., 1998; Kim et al., 1999; Shintani et al., 1999; Tanida et al., 1999). After conjugation of Apg12 to Apg5, the Apg16 protein binds Apg5 noncovalently and dimerizes to link a pair of conjugates (Mizushima et al., 1999). Mutations in any one of these components result in the failure of autophagic vesicle formation/completion. Once completed, autophagosome docking and fusion with the vacuole requires predicted vesicle fusion machinery, including the homologues of syntaxin (Vam3; Darsow et al., 1997), SNAP-25 (Vam7; Sato et al., 1998), and a rab-GTPase (Ypt7; Kim et al., 1999), as well as the class C Vps protein complex (Rieder and Emr, 1997; Scott et al., 1997; Sato et al., 2000; Seals et al., 2000). Successful fusion of the autophagosome with the vacuole membrane releases the single-membrane autophagic body into the vacuole lumen (Takeshige et al., 1992; Baba et al., 1994, 1995). Breakdown of these vesicles, which exposes the autophagic cargo for digestion and recycling, requires the acidification of the vacuole (Nakamura et al., 1997), as well as the vacuolar protease Prb1 (Takeshige et al., 1992) and the putative lipase Cvt17 (Scott et al., 1997; Teter et al., 2001).

The majority of the components required for starvation-induced macroautophagy are also employed to transport selective cargo. Under nutrient-rich conditions, the cytoplasm to vacuole targeting (Cvt) pathway transports the resident hydrolase aminopeptidase I (API) to the vacuole (Klionsky et al., 1992). The overlap in transport machinery between autophagy and the Cvt pathway reflects the mechanistic similarities between the two pathways (Harding et al., 1996; Scott et al., 1996). Precursor API (prAPI) is packaged in double-membrane vesicles (Cvt vesicles) that are substantially smaller than autophagosomes and appear to exclude bulk cytoplasm (Baba et al., 1997; Scott et al., 1997). Completed Cvt vesicles fuse with the vacuole and release a single-membrane vesicle (Cvt body) into the vacuolar lumen (Scott et al., 1997). The breakdown of the Cvt body releases prAPI into its resident lumenal environment, where it is proteolytically processed to the mature form (Klionsky et al., 1992).

In addition to the Cvt pathway, macroautophagy machinery is essential for the selective delivery of excess peroxisomes to the vacuole by the related pexophagy pathway (for review see Kim et al., 2000). Defects in autophagy components compromise peroxisome degradation in *S. cerevisiae* (Hutchins et al., 1999). In the methylotrophic yeast Pichia pastoris, vacuole-mediated degradation of peroxisomes after glucose adaptation utilizes a microperoxisomal mechanism in which designated excess peroxisomes are directly engulfed by the vacuole (Tuttle and Dunn, 1995; Sakai et al., 1998). Despite the difference in the site of sequestration, microperoxisomal pexophagy also clearly requires autophagic machinery as an increasing number of *P. pastoris* homologues of autophagy components are being identified (Yuan et al., 1999).

A key question that remains to be answered is what differentiates nonselective bulk cargo from the specifically targeted cargo, such as prAPI and peroxisomes? Indiscriminate packaging of physiologically critical organelles would result in severe cellular dysfunction. On the other hand, maintaining excess organelles would be energetically wasteful, whereas the failure to sequester and degrade nonfunctional or malfunctioning organelles could result in cell death. To provide such specificity, additional factors are likely required to mediate the sequestration of particular cargo.

In this study, we have identified Cvt9 in *S. cerevisiae* and Gsa9 in *P. pastoris*, which are structural and functional homologues. Cvt9 was identified by its requirement for vacuole uptake of prAPI, and Gsa9 by its role in the turnover of peroxisomal enzymes. Although both Cvt9 and Gsa9 are required for the selective uptake of peroxisomes into the yeast vacuole for degradation, neither protein is essential for nonselective macroautophagy induced by nitrogen starvation. These proteins are associated with a unique membrane compartment located in a perivacuolar region of the cell. Our biochemical and morphological results suggest that Cvt9 and Gsa9 function to selectively sequester cytoplasmic proteins and organelles for degradation within the vacuole.

---

*Abbreviations used in this paper: ALP, alkaline phosphatase; AOX, alcohol oxidase; API, aminopeptidase I; BFP, blue fluorescent protein; Cvt, cytoplasm to vacuole targeting; GFP, green fluorescent protein; HA, hemagglutinin; PGK, phosphoglycerate kinase; prAPI, precursor API; SMD, synthetic minimal medium; td, temperature degron; YNB, yeast nitrogen base.*
Materials and Methods

Strains

The yeast strains used in this study are listed in Table I.

Growth Media

*S. cerevisiae* strains were grown in synthetic minimal medium (SMD: 0.67% yeast nitrogen base [YNB], 2% glucose, and auxotrophic amino acids and vitamins as needed). Starvation experiments were carried out in SD-(0.17% YNB without ammonium sulfate or amino acids, and 2% glucose). Peroxisomes were induced by growth in methanol (YNM: 0.67% YNB, 0.4 mg/ml of glycine, 0.1% Tween 40, and 0.1% oleic acid). Peroxisomes were induced by growth in oleic acid medium (YTO: 0.67% YNB, 0.1% Tween 40, and 0.1% oleic acid). Starvation experiments were carried out in SD-N (0.17% YNB without ammonium sulfate or amino acids, and 2% glucose). Peroxisomes were induced by growth in methanol (YNM: 0.67% YNB, 0.4 mg/ml of glycine, 0.1% Tween 40, and 0.1% oleic acid).

Antibodies/Antiserum

Rabbit antiserum against Cv9 peptides corresponding to amino acids 356–383 and 534–547 (Multiple Peptide Systems) were generated as described previously (Klionsky et al., 1992). Antibodies to API, Fox3, and Apg9 have been described (Klionsky et al., 1992; Hutchins et al., 1999; Noda et al., 2000). Antiserum against phosphoglycerate kinase (PGK) and Pep12 were provided by Dr. Jeremy Thorner (University of California at Berkeley, Berkeley, CA; Baum et al., 1978) and Dr. Scott Emr (University of California at San Diego, La Jolla, CA; Becherer et al., 1996), respectively. Mouse monoclonal antibodies to Dpm1 and Pho8 were from Molecular Probes. Antiseras and antibodies to the hemaggulatin (HA) and m6-epitopes were from Covance Research Products, Inc.

Materials

Reagents are identical to those described previously (Kim et al., 1999; Noda et al., 2000). The copper-inducible promoter-based vectors, pCu416, pCu414, and pCu426, were gifts from Dr. Dennis J. Thiele (University of Michigan, Ann Arbor, MI; Labbé and Thiele, 1999). The pREMI vector was a gift from Dr. Benjamin S. Glick (University of Chicago, Chicago, IL). The pGFP(416)SK and pGFP(426)SK vectors for expressing green fluorescent protein (GFP) fusions were a gift from Dr. Tom Vida (University of Texas Health Sciences Center, Houston, TX). The temperature de‐
gon (td) plasmid was a gift from Dr. Jürgen Dohmen (University of Cologne, Cologne, Germany; Dohmen et al., 1994).

Cloning CVT9

The cv9 strain AHY96 (Harding et al., 1996) was transformed with a YCp50-based yeast genomic library (Rose et al., 1987). The transformed colonies were subjected to a nitrogen starvation screen as described previously (Kim et al., 1999). After 30 d, colonies grown on the first 100 survivors examined contained mature API. The complementing plasmid was recovered and sequenced using pBR322 oligonucleotides. Partial sequences were entered into the Saccharomyces genome database (SGD; http://genome-website.stanford.edu/Saccharomyces/) and a 20.9-kb region on chromosome XVI was identified. A 4,812-bp fragment that complemented the prAPI maturation defect was introduced into pRS414 and pRS424 by using the endogenous Spel-BamHI restrictions sites, resulting in the pCVT9(414) and pCVT9(424) clones. These clones contain the 3,537-bp CVT9 ORF (YPR041C) plus 585 bp of the 5' end and 690 bp of the 3' noncoding sequences. Alternatively, the CVT9 gene was amplified from genomic DNA by PCR and cloned into the vector pBluescript to generate pBS(CVT9). A 4.8-kb Spel-BamHI fragment containing the CVT9 gene from pBS(CVT9) was cloned into pRS313 to generate p313(CVT9).

Isolation of gsa9 Mutants and Cloning GSA9

P. pastoris gsa mutants were isolated after the restriction enzyme–mediated integration of a 2.0-kb preMI plasmid which contained the Col El origin of replication and the Zeocin resistance gene under the control of the TEFI promoter from *S. cerevisiae* and the EM7 promoter of *E. coli*. Details of the mutagenesis procedure will be described elsewhere. Three different gsa mutants had a disruption of the GSA9 gene.

### Table I. Strains Used in This Study

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. cerevisiae strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KA311A</td>
<td>MATa his3 leu2 trpl ura3</td>
<td>Irie et al., 1993</td>
</tr>
<tr>
<td>YYY36</td>
<td>KA311A apq1 Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YYK107</td>
<td>KA311A cvt9Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>SEY6210</td>
<td>MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel GAL</td>
<td>Robinson et al., 1988</td>
</tr>
<tr>
<td>VDY101</td>
<td>SEY6210 apq7Δ::LEU2</td>
<td>Kim et al., 1999</td>
</tr>
<tr>
<td>JKY007</td>
<td>SEY6210 apq9Δ::HIS3</td>
<td>Noda et al., 2000</td>
</tr>
<tr>
<td>THY119</td>
<td>SEY6210 cvt3-1</td>
<td>Harding et al., 1995</td>
</tr>
<tr>
<td>AHY001</td>
<td>SEY6210 cvt9Δ::HIS3</td>
<td>Harding et al., 1995</td>
</tr>
<tr>
<td>AHY96</td>
<td>SEY6210 cvt9-1</td>
<td>Harding et al., 1996</td>
</tr>
<tr>
<td>JGyt9d</td>
<td>SEY6210 cvt9Δ::CVT9-DHFR-Ub-HA</td>
<td>Harding et al., 1996</td>
</tr>
<tr>
<td>AHY1468</td>
<td>SEY6210 cvt10-1</td>
<td>Harding et al., 1996</td>
</tr>
<tr>
<td>TVY1</td>
<td>SEY6210 pep4Δ::LEU2</td>
<td>Gerhardt et al., 1998</td>
</tr>
<tr>
<td>WSY99</td>
<td>SEY6210 ypp7Δ::HIS3</td>
<td>Wurmser and Emr, 1998</td>
</tr>
<tr>
<td>TN121</td>
<td>MATa leu2-3,112 trp1 ura3-52 pho8Δ::pho8Δ60 pho13Δ::URA3</td>
<td>Noda et al., 1995</td>
</tr>
<tr>
<td>AHY021</td>
<td>TN121 cvt9Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>TN125</td>
<td>MATa ade2 his3 leu2 lys2 trp1 ura3 pho8Δ::pho8Δ60</td>
<td>Noda and Ohsumi, 1998</td>
</tr>
<tr>
<td>YYK126</td>
<td>TN125 apg1 Δ::LEU2</td>
<td>Kamada et al., 2000</td>
</tr>
<tr>
<td>YYK127</td>
<td>TN125 cvt9Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td><strong>P. pastoris strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS115</td>
<td>his4</td>
<td>Cregg et al., 1985</td>
</tr>
<tr>
<td>WDY7</td>
<td>his4 gsa7</td>
<td>Yuan et al., 1999</td>
</tr>
<tr>
<td>R8</td>
<td>GS115 gsa9-2::Zeocin</td>
<td>This study</td>
</tr>
<tr>
<td>WDY26</td>
<td>GS115 his4::pWD11 (P<em>goa</em> HA-GSA9, HIS4)</td>
<td>This study</td>
</tr>
<tr>
<td>DMM1</td>
<td>GS115::pDM1 (P<em>ox2</em>GFP-SKL, Zeocin)</td>
<td>This study</td>
</tr>
<tr>
<td>ANB23</td>
<td>DMM1 his4::pPS64 (P<em>Gao</em> GFP-HA-GSA9, HIS4)</td>
<td>This study</td>
</tr>
<tr>
<td>SMD163</td>
<td>his4 pep4 prb1</td>
<td>Tuttle and Dunn, 1995</td>
</tr>
<tr>
<td>PPF1</td>
<td>his4 arg4</td>
<td>Yuan et al., 1997</td>
</tr>
<tr>
<td>WDK09</td>
<td>PPF1 gsa9Δ::ARG4</td>
<td>This study</td>
</tr>
<tr>
<td>ANB12</td>
<td>WDK09 his4::pPS64 (P<em>Gao</em>GFP-HA-GSA9, HIS4)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Published April 16, 2001

Kim et al. Cv9/Gsa9 Functions in the Cvt and Pexophagy Pathways

383

Downloaded from on April 19, 2017
gsa9-1 cells had the pREMI vector inserted between Q1068 and Y1069, gsa9-2 between M83 and S84, and gsa9-3 between R1291 and A1292. Using the different genomic DNA fragments from the three different allelic mutants, we were able to completely assemble the GSA9 gene as well as 700 bp of the 5’ and 600 bp of the 3’ noncoding regions. The genomic sequence of GSA9 reported in this paper has been deposited in the National Center for Biotechnology Information (EMBL/GenBank/DDBJ accession number AF309870).

Disruption of CVT9 and GSA9

The chromosomal CVT9 locus was deleted by a PCR-based, one-step procedure (Noda et al., 2000). The auxotrophic HIS3 gene was amplified from the pRS313 vector using oligonucleotides that contained vector sequences outside of the HIS3 marker flanked by CVT9 sequences that encode regions at the beginning and end of the CVT9 ORF. The PCR product was used to transform yeast strain SEY6210 and the His+ product was used to transform yeast strain SEY6210 and the His+ transformants were screened for prAPI accumulation to identify CVT9 knockouts. The cvt9Δ strain was confirmed by tetrad analysis as described previously (Kim et al., 1999). Alternatively, a 1.1-kb BglII-EcoRV fragment from within the CVT9 gene in the plasmid pUC19 was replaced with the LEU2 gene. This plasmid was digested with HindIII and transformed into the TN125 yeast strain to make strain YYK127. Transformants were selected on the appropriate auxotrophic plates and screened by immunoblot using antiserum to API.

To disrupt GSA9, the S. cerevisiae ARG4 gene with its promoter was amplified by PCR using primers that contained 52 bp of 5’ and 3’ regions of GSA9 and 27 bp of the ARG4 gene. The PCR product was directly used to transform PPF1 cells. The resulting Arg+ clones were replica-plated to YNM plates and their inability to degrade alcohol oxidase (AOX) during glucose adaptation was determined by direct colony assay as described below. The site of ARG4 insertion within WDK09 resulting in the depletion of >90% of the GSA9 gene was verified by PCR.

Plasmid Constructions

**Generation of Epitope-tagged Cvt9.** To epitope-tag Cvt9, the BamHI site in p313[3CVT9] (Sears et al., 1998) was linearized using restriction endonuclease XhoI sites of pIB2 (Sears et al., 1998). This plasmid, pWD11, was linearized by cutting within the GAPDH promoter was linearized by cutting within the HIS4 gene and used to transform GS115 cells.

**Disruption of CVT9 and GSA9.** The chromosomal CVT9 locus was deleted by a PCR-based, one-step procedure (Noda et al., 2000). The auxotrophic HIS3 gene was amplified from the pRS313 vector using oligonucleotides that contained vector sequences outside of the HIS3 marker flanked by CVT9 sequences that encode regions at the beginning and end of the CVT9 ORF. The PCR product was used to transform yeast strain SEY6210 and the His+ transformants were screened for prAPI accumulation to identify CVT9 knockouts. The cvt9Δ strain was confirmed by tetrad analysis as described previously (Kim et al., 1999). Alternatively, a 1.1-kb BglII-EcoRV fragment from within the CVT9 gene in the plasmid pUC19 was replaced with the LEU2 gene. This plasmid was digested with HindIII and transformed into the TN125 yeast strain to make strain YYK127. Transformants were selected on the appropriate auxotrophic plates and screened by immunoblot using antiserum to API.

To disrupt GSA9, the S. cerevisiae ARG4 gene with its promoter was amplified by PCR using primers that contained 52 bp of 5’ and 3’ regions of GSA9 and 27 bp of the ARG4 gene. The PCR product was directly used to transform PPF1 cells. The resulting Arg+ clones were replica-plated to YNM plates and their inability to degrade alcohol oxidase (AOX) during glucose adaptation was determined by direct colony assay as described below. The site of ARG4 insertion within WDK09 resulting in the depletion of >90% of the GSA9 gene was verified by PCR.

**Two-Hybrid Analysis.** A two-hybrid screen was carried out using a truncated form of the APG1 ORF lacking the NH2-terminal eight amino acids as the bait plasmid as described (Kamada et al., 2000).

**Nitrogen Survival Assay.** Nitrogen starvation analysis was done as described previously (Scott et al., 1996).

**Alkaline Phosphatase Enzyme Assay.** Induction of autophagy was estimated as activity of mutated alkaline phosphatase (ALP; Pho860p) with α-naphthyl phosphate (Sigma-Aldrich) as substrate as described (Noda and Ohsumi, 1998).

**Measurements of Protein and Peroxisome Degradation.** The degradation of peroxisomes in S. cerevisiae was determined by the loss of thiolase (Fox3) as described previously (Hutchins et al., 1999). The degradation of peroxisomes in P. pastoris was evaluated by measuring the loss of AOX during glucose adaptation. Samples were assayed for AOX activity as described previously (Yuan et al., 1999) at 0 and 6 h after shifting to glucose. The degradation of cellular proteins during nitrogen starvation was performed as described previously (Yuan et al., 1999).

**Cell Labeling, Immunoprecipitations, and Immunoblot Analysis.** Radioisotopic labeling of cells, immunoprecipitations, and immunoblotting were performed as described previously (Noda et al., 2000).

**Protease Protection and Membrane Floatation Assays.** Protease sensitivity of prAPI in the cvt9Δ and prp7A strains and membrane flotation experiments were performed as described previously (Noda et al., 2000).

**Subcellular Fractionations.** For subcellular fractionation experiments, cvt9Δ cells transformed with the pCuCVT9(416) plasmid were incubated with 50 μM CuSO4 for 1 h at midlog stage and then spheroplasted as described previously (Kim et al., 1999). Spheroplasts were subjected to differential lysis in PS200 lysis buffer A (20 mM PIPES, pH 6.8, 200 mM sorbitol, 5 mM MgCl2) containing the CompleteTM EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals) and 1 μM PMSF. After a preclearing step at 100 g, 4°C, 5 min, the lysate was subjected to low-speed centrifugation at 16,000 g (13,000 rpm) in an Eppendorf 5415D microcentrifuge for 5 min at 4°C. The low-speed supernatant fraction (S13) was further resolved into high-speed supernatant and pellet fractions (S100 and P100, respectively) by centrifugation at 100,000 g for 20 min at 4°C in a centrifuge (TL100) using a TLA100.4 rotor (both from Beckman Coulter).

For the biochemical characterization of Cvt9 membrane association, the total lysate was mixed with an equal volume of 1% Triton X-100 (in PS200), 6 μM ura (in PS200), 0.1 M Na2CO3, pH 10.5 (in 200 mM sorbitol), or 1.0 M salt wash (0.67 M KOAc, 0.3 M KCl). After a 5-min incubation at 25°C, the treated lysates were separated into high speed supernatant and pellet fractions by centrifugation at 100,000 g for 20 min at 4°C.

To assess prAPI binding, cvt9Δ spheroplasts were lysed in PS200 containing 1, 2, or 5 mM MgCl2 and separated into low-speed supernatant and pellet fractions by centrifugation at 2,300 g (5,000 rpm) in an Eppendorf 5415D microcentrifuge for 5 min at 25°C. All fractionated samples were subjected to immunoblot analysis.

**Optiprep Gradient Analysis.** For analysis of Cvt9 by Optiprep gradients, cvt9Δ cells transformed with pCuCVT9(416) were incubated with 50 μM CuSO4 for 1 h to induce Cvt9 expression. At midlog stage the cells were spheroplasted and lysed by gradient buffer (PS200 containing 1 mM MgCl2, 1 mM DTT, 1 mM EDTA, and CompleteTM EDTA-free protease inhibitor cocktail). After a pre-clearing step at 100 g at 4°C for 5 min in an Eppendorf 5415D microcentrifuge, the lysate was separated into high-speed supernatant and pellet fractions by direct centrifugation at 100,000 g for 20 min at 4°C using a
TAL100.4 rotor. The total membrane fraction was resuspended in gradient buffer and loaded to the top of a 10 ml Optiprep linear gradient (10–66%) and centrifuged in an SW41 rotor at 100,000 g for 16 h at 4°C. 14 fractions were collected and analyzed by immunoblotting.

**Microscopy**

**Confocal Microscopy.** The cvt9Δ cells transformed with pCtGFP/CVT9(416) were induced with 10 μM CuSO4 for 2 h, followed by labeling of vacuoles with FM 4-64 and viewing the cells using a confocal microscope (IRM, Leica) as described previously (Kim et al., 1999).

**Fluorescence Microscopy.** Cells expressing GFP/HA-Gsa9 were grown in glucose or methanol for 2–24 h. FM 4-64 (20 μg/ml) was added and the cells were incubated for an additional 16 h. Cells grown on methanol were transferred to glucose medium for 1–3 h. The cells were then washed and examined immediately using an Axiohot fluorescence microscope (ZEISS). Image capture was done using a SPOT digital camera (Diagnostic Instruments, Inc.) with Adobe Photoshop® software.

**Electron Microscopy.** Cells were grown for 40 h in medium supplemented with 0.5% methanol and then transferred to medium containing 2% glucose for 2 h. The cells were washed with distilled water and fixed in 1.5% KMnO4. The cells were then dehydrated, embedded in Epon 812, and sectioned for viewing on a JEOL 100CX transmission electron microscope (Yuan et al., 1999).

**Coimmunoprecipitation of Cvt9 and Apg1**

Cells expressing the NH2-terminally myc-tagged Cvt9 and HA-tagged Apg1 were grown to midlog phase in YPD and spheroplasts as described previously (Kamada et al., 2000). The spheroplasts were lysed with ice-chilled Tween lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.4 mM Na2VO4, 50 mM KF, 20 μg/ml leupeptin, 20 μg/ml benzamidine, 10 μg/ml pepstatin A, 40 μg/ml aprotinin, 1 mM PMSF, and 0.5% Tween-20). The lysate was centrifuged at 4,000 g for 5 min and precleared with 20 μl of protein G-Sepharose (50% suspension) for 30 min at 4°C. The cell lysate (500 μl) was incubated with or without 3 μl of anti-Apg1 antisera for 2.5 h at 4°C. Immunocomplexes were collected on protein G-Sepharose beads and washed three times with Tween lysis buffer. The resultant immunocomplexes were subjected to 7.5% SDS-PAGE and analyzed further by immunoblotting with anti-myc ascite (9E10) to detect myc-tagged Cvt9 or with anti-HA ascite (16B12) to detect HA-tagged Apg1. Coimmunoprecipitation of 3×HA-Cvt9 and 3×myc-Cvt9 was performed in essentially the same manner.

**Results**

The *apg* and *cvt* mutants share a significant genetic overlap, indicating a mechanistic convergence between autophagy and the Cvt pathway (for review see Kim and Klionsky, 2000). Therefore, the strong sensitivity to nitrogen starvation displayed by most autophagy mutants has been used as a phenotypic screen to isolate the complementing plasmids for the majority of the *cvt* and *apg* mutants cloned thus far. Although *cvt9* exhibited only marginal sensitivity to nitrogen starvation, we nevertheless used the starvation screen in an attempt to enrich for *cvt9* cells containing the *CVT9*-complementing plasmid. The *cvt9* mutant was transformed with a yeast genomic library and subjected to nitrogen starvation. After 30 d of this regime, 1 out of the first 100 surviving colonies screened exhibited a reversal of the prAPI accumulation phenotype. The plasmid was isolated and the complementing ORF was determined to be YPR049C, which was named *CVT9*. Further genetic analysis confirmed that *CVT9* mapped to the same locus as the original *cvt9* mutation (see Materials and Methods).

**Cvt9 Is Required for Cvt Transport but Not Autophagy**

The *CVT9* single-copy plasmid rescued the prAPI processing defect in the *cvt9* mutant strain (Fig. 1 A), although overexpression of Cvt9 using a multicopy *CVT9* plasmid resulted in a moderate accumulation of prAPI. Although the *CVT9* clone was isolated using the nitrogen starvation strategy, the viability of the *cvt9*-null strain remained relatively robust in starvation conditions when compared with a typical mutant in the autophagy pathway (e.g., *cvt10*/*apg1*; Scott et al., 1996; Fig. 1 B). Viability under starvation conditions reflects a capacity for autophagy, indicating an ability to recycle cytosolic material after delivery to the vacuole. Therefore, we next determined if the resistance to nitrogen starvation exhibited by the *cvt9Δ* strain reflects its ability to carry out autophagy.

The proteolytic processing of prAPI to its mature form in the vacuole provides a useful marker to assess the proper function of the Cvt pathway in nutrient-rich conditions and the autophagy pathway under starvation conditions (Scott et al., 1996). In mutants that exhibit both Cvt and autophagy defects (e.g., *apg7Δ*; Kim et al., 1999; Fig. 1 C), prAPI accumulation persists in nutrient-rich as well as starvation conditions. However, if a mutant displays a defect only in Cvt transport (e.g., *cvt3*; Harding et al., 1995; Scott et al., 1996; Fig. 1 C), then the accumulated prAPI phenotype can be rescued by diverting prAPI transport from a defective Cvt pathway to a functional autophagy pathway after shifting cells to starvation conditions. In *cvt9Δ*, the prAPI delivery defect observed in nutrient-rich conditions could be circumvented by a shift to nitrogen starvation conditions, suggesting that a functional autophagy pathway exists to transport prAPI to the vacuole (Fig. 1 C).

To further demonstrate the competence for autophagic uptake in *cvt9Δ*, we examined the recombinant cytosolic marker protein Pho8Δ60. Truncating the NH2-terminal transmembrane domain in the vacuolar hydrolase (ALP or Pho8) results in its cytosolic localization (Noda et al., 1995). This modified protein is delivered to the vacuole solely by autophagy. Proteolytic cleavage of the Pho8Δ60 propeptide in the vacuolar lumen generates the active form of the enzyme, which can be detected by an activity assay (Fig. 1 D) or by immunoblot analysis (data not shown). Wild-type, *apg1Δ*, and *cvt9Δ* strains were transformed with an integrating plasmid that replaced the *PHO8* gene with *pho8Δ60*. Cells were vegetatively grown to midlog phase and then shifted to nitrogen starvation conditions to induce autophagy. After 4 h in SD-N, vacuolar ALP activity was measured (Fig. 1 D). The ALP activity in the wild-type strain increased significantly in SD-N, indicating that Pho8Δ60 delivery and subsequent processing to its enzymatically active form occurred in the vacuole. As expected, the shift to SD-N did not increase the ALP activity in the autophagy-defective strain *apg1Δ*. However, in *cvt9Δ*, incubation in nitrogen starvation conditions caused a strong increase in ALP activity, further demonstrating that Cvt9 is not essential for autophagic transport.

The fusion of double membrane Cvt vesicles or autophagosomes with the vacuole releases the single-membrane Cvt or autophagic body into the vacuole lumen, respectively (Baba et al., 1997). The subsequent break-down of these Cvt or autophagic bodies is dependent on resident hydrolases, as treatment with protease inhibitors such as PMSF will prevent the breakdown of these sin-
Cvt9 is essential for the Cvt pathway but not for autophagy. (A) CVT9 complements the prAPI transport defect in the cvt9 mutants. Wild-type (WT; SEY6210), cvt9 (AHY96), and cvt9Δ (AHY001) strains transformed with either the centromeric (CEN) or multicopy (2μ) CVT9 plasmid (pCVT9) were grown to midlog phase in SMD. Protein extracts were prepared and analyzed by immunoblots using antiserum to API. The positions of precursor and mature API are indicated. (B) The cvt9Δ strain is largely resistant to nitrogen starvation conditions. Wild-type (SEY6210), cvt9Δ (AHY001), and cvt10/apg1 (AHY1468) cells were grown to midlog phase in SMD and transferred to SD-N as described in Materials and Methods. Aliquots were removed at the indicated times and spread onto YPD plates in triplicate. Numbers of viable colonies were determined after 2–3 d. (C) The accumulation of prAPI in cvt9Δ can be partially reversed by nitrogen starvation. The apg7Δ (VDY101), cvt3 (THY119), and cvt9Δ (AHY001) strains were grown to midlog phase and shifted to SD-N. Protein extracts were prepared at the indicated times and analyzed by immunoblots with antiserum to API. (D) ALP activity assay for autophagy. Wild-type (TN125), apg1Δ (YYK126), and cvt9Δ (YYK127) cells expressing Pho8Δ60 were shifted from nutrient-rich medium (white bars) to SD-N nitrogen-depleted medium (black bars) for 4 h. The level of autophagy induction was determined by an ALP activity assay as described in Materials and Methods. The graph is the average of three experiments. The cvt9Δ strain is not defective in the vacuolar delivery of the autophagy marker Pho8Δ60 during starvation conditions. (E) Cvt9 is not required for the formation of autophagosomes. The cvt9Δ (YYK127) strain was grown in nutrient-rich (YPD) and nitrogen starvation (SD-N) conditions supplemented with the protease inhibitor PMSF and examined by electron microscopy as described in Materials and Methods. In YPD, Cvt complexes (indicated by an arrow) could be detected in the cytoplasm. In SD-N, cvt9Δ cells accumulated autophagic bodies in the vacuole when treated with PMSF.
and pellet (P) fractions by centrifugation at 2,300 rpm for 5 min. An aliquot was removed for the total lysate control (T). The pellet fraction (P) was resuspended in 15% Ficoll-400 and added to the bottom of a step gradient of 13 and 2% Ficoll-400. The step gradient was centrifuged at 16,000 rpm for 10 min. Membrane-containing float (F), nonfloat (NF), and pellet (P) fractions were immunoprecipitated with antiserum to API as described in Materials and Methods. (A) prAPI membrane binding is destabilized in the cvt9Δ mutant. The cvt9Δ (AHY001) and apg9Δ (JKY007) strains were grown to midlog phase and converted into spheroplasts. The spheroplasts were lysed in an osmotic lysis buffer supplemented with 0, 1, 2, or 5 mM MgCl2. The total lysate (T) was separated into low-speed supernatant (S) and pellet (P) fractions by centrifugation at 2,300 rpm for 5 min. An aliquot was removed for the total lysate control (T). The pellet fraction (P) was resuspended in 15% Ficoll-400 and added to the bottom of a step gradient of 13 and 2% Ficoll-400. The step gradient was centrifuged at 16,000 rpm for 10 min. Membrane-containing float (F), nonfloat (NF), and pellet (P) fractions were immunoprecipitated with antiserum to API as described in Materials and Methods. (B) The temperature-conditional cvt9td strain is protease accessible. Spheroplasts isolated from cvt9td and ypt7Δ cells were pulse-labeled for 10 min and chased for 30 min at 37°C. The labeled spheroplasts were then osmotically lysed and separated into low-speed supernatant (S) and pellet (P) fractions after a 2,300-g centrifugation step. The pellet fractions were subjected to protease treatment in the absence or presence of 0.2% Triton X-100 as described in Materials and Methods. (D) prAPI in the cvt9Δ strain associates with a floatable membrane fraction. Spheroplasts of apg9Δts and cvt9Δ cells were osmotically lysed and separated into supernatant (S) and pellet (P) fractions by centrifugation at 2,300 g for 5 min. An aliquot was removed for the total lysate control (T). The pellet fraction (P) was resuspended in 15% Ficoll-400 and added to the bottom of a step gradient of 13 and 2% Ficoll-400. The step gradients were centrifuged at 16,000 g for 10 min. Membrane-containing float (F), nonfloat (NF), and pellet (P) fractions were immunoprecipitated with antiserum to API as described in Materials and Methods.
prAPI remained largely in the supernatant fraction even at 2 mM MgCl₂, with significant prAPI membrane binding occurring only at 5 mM MgCl₂. These results indicate that although Cvt9 is not essential for prAPI membrane bind-
ing, the deletion of CVT9 destabilizes the interactions between prAPI oligomers and the membrane structure to which they bind.

**Cvt9 Is Required at the Stage of Cvt Vesicle Formation/Completion**

The permanent loss of function in the cvt9Δ mutant may result in indirect, secondary phenotypes on the Cvt pathway. To determine the direct effects of Cvt9 loss of function, we generated a temperature-sensitive form of Cvt9 by constructing a chimera of Cvt9 linked to the COOH terminus of Ub-DHFR, resulting in the Cvt9td fusion protein (Dohmen et al., 1994). The expression of Cvt9td was placed under the copper-inducible CUP1 promoter. At permissive temperature (30°C), the strain (JGY9td) expressing the Cvt9td protein was fully functional for the Cvt pathway and displayed prAPI maturation kinetics that were similar to the wild-type strain (Fig. 2 B). A shift to nonpermissive temperature caused the rapid misfolding and degradation of Cvt9td by proteasome-mediated proteolysis (data not shown; Dohmen et al., 1994). As a consequence, prAPI import was tightly blocked in the cvt9td mutant at the nonpermissive temperature, whereas prAPI maturation kinetics remained unaffected in the wild-type strain (Fig. 2 B). The immediate defect in prAPI processing caused by the thermal inactivation of Cvt9td suggests that Cvt9 acts directly in the Cvt pathway.

To determine the step of the pathway in which Cvt9 function is required, we analyzed the state of prAPI in the cvt9td strain by protease sensitivity and membrane flotation assays. Cvt9td expression was induced at midlog phase for 1 h with 1 μM CuSO₄ before spheroplasting. The cvt9td spheroplasts were radioactively labeled for 5 min followed by a nonradioactive chase reaction for 30 min at nonpermissive temperature. The labeled spheroplasts were then lysed osmotically and separated into low-speed supernatant and pellet fractions as described in Materials and Methods. The prAPI-containing pellet fractions were treated with exogenous proteinase K in the presence or absence of detergent and then immunoprecipitated with anti-serum to Cvt9 and antibodies to the cytosolic marker protein PGK. The supernatant and pellet fractions were also immunoprecipitated with antisera to the cytosolic marker protein PGK to assess the osmotic spheroplast lysis efficiency. The protease sensitivity assay indicates that at nonpermissive temperature, prAPI in the cvt9td strain was accessible to exogenous proteases even in the absence of detergent treatment (Fig. 2 C). The small amount of protease-resistant prAPI in the cvt9td strain probably reflects incomplete spheroplast lysis based on the appearance of a small amount of PGK in the pellet fraction. Nevertheless, the protease-protection assay of cvt9td at nonpermissive temperature suggests that Cvt vesicles had not yet formed to enclose prAPI in this strain. The ypt7Δ mutant was used as the control strain. Ypt7 is a rab guanine nucleotide exchange factor for Cvt vesicle fusion with the vacuole, and thus the ypt7Δ strain accumulates prAPI in a protease-protected state (Fig. 2 C; Kim et al., 1999).

In the membrane flotation gradient analysis, membrane-associated proteins in a cell lysate migrate to the top of a step gradient and are collected as the “float” (F) fraction. Soluble proteins remain in the nonfloat (NF) fraction and proteins associated with large protein complexes are recovered in the gradient pellet fraction (P2). The radiola-

---

**Figure 3.** Characterization of Cvt9. (A) Cvt9 forms a homo-dimer. The wild-type (KA311A) strain was transformed with a plasmid encoding myc-tagged Cvt9 with or without a second plasmid for HA-tagged Cvt9. Cell lysates were prepared as described in Materials and Methods and immunoprecipitated (IP) with antisera to HA (lanes 1 and 3) or buffer control (lanes 2 and 4). The immunoprecipitates were then immunoblotted with antiserum to HA (lanes 1 and 3) or buffer control (lanes 2 and 4). The immunoprecipitates were then immunoblotted with antisera to Cvt9 and antibodies to the ER integral membrane protein Dpm1.

(B) Cvt9 subcellular fractionation pattern. The cvt9Δ (AHY001) strain was transformed with a CVT9 plasmid behind the CUP1 copper-inducible promoter (pCuCVT9[416]). Expression was induced with 50 μM CuSO₄ for 1 h before spheroplasting. The spheroplasts were osmotically lysed (see Materials and Methods). After a preclearing centrifugation step at 100,000 g for 5 min to remove unlysed spheroplasts, the total lysate (T) was separated into low speed supernatant (S13) and pellet (P13) fractions. The S13 fraction was further separated into 100,000 g supernatant (S100) and pellet (P100) fractions.

(C) Cvt9 is a peripheral membrane protein that is resistant to protease. A total lysate was prepared as in B and resuspended in osmotic lysis buffer only or containing 1% Triton X-100, 6 M urea, 0.1 M Na₂CO₃, pH 10.5, or a 1.0 M salt wash (0.67 M KOAc, 0.3 M KCl). The treated lysates were separated into total membrane (P) and supernatant (S) fractions by centrifugation at 100,000 g for 20 min at 4°C. The samples were analyzed by immunoblots with antisera to Cvt9 and antibodies to the ER integral membrane protein Dpm1.
beled prAPI pellet fraction derived from the lysate of the cvt9Δ strain at nonpermissive temperature was loaded on the bottom of a step gradient and subjected to centrifugation. The majority of prAPI from the cvt9Δ strain was recovered in the float fraction, indicating that prAPI associates with a floatable membrane source (Fig. 2 D). As a positive control for the flotation gradient analysis, we examined the prAPI pellet fraction derived from a temperature-conditional allele of apg9 (apg9ts). Consistent with our recent study on Apg9, prAPI from the apg9ts strain as-

Figure 4. Cvt9 is localized to a perivacuolar compartment. (A) Localization pattern of GFPCvt9. The cvt9Δ (AHY001) strain was transformed with a plasmid encoding a GFPCvt9 fusion protein behind the CUP1 promoter (pCuGFPCVT9). Expression of GFPCvt9 was induced with 10 μM CuSO4 for 2 h, followed by labeling of the vacuoles with FM 4-64 (see Materials and Methods). Images were taken with a Leica IRM confocal microscope. GFPCvt9 mostly localizes to intense perivacuolar, punctate structures. (B and C) Subcellular localization of Cvt9 by Optiprep density gradients. The cvt9Δ strain was transformed with pCuCVT9(416) and incubated with 50 μM CuSO4 at midlog growth stage to induce Cvt9 expression. The cells were converted to spheroplasts and lysed in gradient lysis buffer (see Materials and Methods). A total membrane fraction was isolated by centrifugation at 100,000 g for 20 min and loaded to the top of a 10-ml Optiprep linear gradient (0–66%). After centrifugation at 100,000 g for 16 h at 4°C, 14 fractions were collected and analyzed by immunoblots with antiseraum or antibodies to Cvt9 and (B) Pho8 (vacuole), (C) Dpm1 (ER), Kex2 (trans-Golgi Network), and Pep12 (endosome). The immunoblots and the graphs of the densitometry quantitation are from the same gradient but presented separately for clarity.
Figure 5. Cvt9 physically associates with Apg1. The apgiΔ strain (YYK36) was transformed with a plasmid encoding myc-tagged Cvt9 (p[myc-CVT9]) with or without an additional plasmid encoding Apg1 (p[APG1]). The cell lysates were prepared as described in Materials and Methods and subjected to immunoprecipitation with anti-Apg1 antiserum (lanes 1, 3, and 5) or buffer only (lanes 2, 4, and 6). The immunoprecipitates (IP) were analyzed by immunoblot with antiserum to myc. The anti-Apg1–precipitated immunocomplex contains myc-tagged Cvt9 (lane 5).

Cvt9 Is an Oligomeric, Coiled Coil, Peripheral Membrane Protein

To further understand Cvt9 function in the Cvt pathway, we next characterized the biosynthesis of the Cvt9 protein. Polyclonal antiserum raised against synthetic Cvt9 peptides recognized a 135-kD band by immunoblot analysis, in agreement with its predicted molecular mass (data not shown). Detection of Cvt9 increased in cvt9Δ cells transformed with the Cvt9 multicycop plasmid and was absent in the cvt9Δ cells, suggesting that the antiserum is specific for the Cvt9 protein. Analysis of the Cvt9 amino acid sequence reveals a significant coiled coil secondary structure between amino acid residues 542 and 851 (http://nightingale.lcs.mit.edu/cgi-bin/score). The P. pastoris homologue, Gsa9, also contains a predicted coiled coil secondary structure motif between amino acids 811 and 1027. Because a common feature of proteins containing a coiled coil domain is the ability to form higher-ordered quaternary structures between amino acid residues 542 and 851 (http://nightingale.lcs.mit.edu/cgi-bin/score). The P. pastoris homologue, Gsa9, also contains a predicted coiled coil secondary structure motif between amino acids 811 and 1027. Because a common feature of proteins containing a coiled coil domain is the ability to form higher-ordered quaternary structures, we investigated the possibility that Cvt9 forms oligomers. Extracts from cells expressing NH2-terminally myc-tagged Cvt9 and HA-tagged Cvt9 were prepared and the immunocomplex containing HA-Cvt9 was immunoprecipitated under native, nonreducing conditions with antiserum to the HA epitope. A subsequent anti-myc epitope immunoblot detected the myc-tagged Cvt9 from the original immunoprecipitated HA–Cvt9 immunocomplex, indicating that myc-Cvt9 associates with HA–Cvt9 (Fig. 3 A). These findings indicate that Cvt9 forms homodimers or potentially larger oligomeric structures.

The subcellular location of Cvt9 was initially examined by subcellular fractionation and differential centrifugation procedures. The cvt9Δ strain was transformed with a plasmid containing CVT9 behind the CUP1 copper regulable promoter (Labbé and Thiele, 1999). Cvt9 expression was induced at midlog phase with 50 μM CuSO4 for 1 h before spheroplasting. The prAPI accumulation in the cvt9Δ strain was completely reversed at this level of Cvt9 expression (data not shown). The spheroplasts were lysed osmotically and separated into low-speed supernatant and pellet fractions (S13 and P13, respectively). The S13 supernatant fraction was further resolved into high-speed supernatant and pellet fractions (S100 and P100, respectively). The majority of Cvt9 fractionated to both the P13 and P100 fractions, whereas only a minor population appeared in the high-speed supernatant fraction (Fig. 3 B). We next investigated the nature of the Cvt9 pellet association (Fig. 3 C). The spheroplasts were lysed osmotically and treated with lysis buffer, Triton X-100, urea, Na2CO3, or a high salt wash as described in Materials and Methods. The treated lysates were then subjected to high-speed centrifugation and separated into total membrane and supernatant fractions. The buffer-only treatment confirmed that the majority of Cvt9 remained bound to a membrane pellet fraction. However, treatment with urea, Na2CO3, and the high-salt wash, conditions that remove peripheral membrane proteins, all stripped a substantial portion of Cvt9 from the membrane pellet into the supernatant fraction. In addition, examination of Cvt9 by membrane flotation gradient analysis indicated that Cvt9 was recovered in the “float” fraction, suggesting that it associated with a floatable membrane (data not shown). Therefore, we propose that Cvt9 is a peripheral membrane protein (Fig. 3 C). In contrast, the integral membrane protein Dpm1 remained in the pellet fraction under all conditions, except when membranes were solubilized with detergent. Interestingly, Cvt9 remained in the pellet fraction after treatment with Triton X-100, suggesting that Cvt9 multimers may form a larger detergent-resistant protein complex.
Kim et al. Cvt9/Gsa9 Functions in the Cvt and Pexophagy Pathways

Distinct from the vacuole. Furthermore, the Cvt9 compartment did not appear to cofractionate with any other resident organelle marker proteins, including Dpm1 (ER), Kex2 (late Golgi apparatus), and Pep12 (endosome) (Fig. 4 C). Taken together, the identity of the perivacuolar compartment to which Cvt9 localizes appears to be distinct from known organelles of the endomembrane system.

Cvt9 Physically Interacts with the Apg1 Kinase

Cvt9 is present in a detergent-resistant form, presumably a protein complex, which localizes to a distinct membrane compartment (Figs. 3 and 4). We next identified an additional factor of the Cvt9 protein complex. A two-hybrid analysis to identify proteins that associate with the autophagy component Apg1 identified Cvt9 as a potential interacting partner (Kamada et al., 2000). The Apg1–Cvt9 interaction was directly supported by a coimmunoprecipitation analysis (Fig. 5). The \( \text{apg1} \) strain was transformed with the plasmid encoding the myc-tagged Cvt9 fusion protein with or without a second plasmid encoding Apg1. Cell extracts were immunoprecipitated with antibody to Apg1 under native, nondenaturing conditions as described in Materials and Methods. The precipitated immunocomplexes were then subjected to a denaturing SDS-PAGE/immunoblot procedure with antibody to the myc epitope. In cells expressing both myc–Cvt9 and Apg1, the immunocomplexes precipitated with the Apg1 antibody also isolated the myc-tagged Cvt9 protein, thus confirming the two-hybrid data that Apg1 and Cvt9 physically interact (Fig. 5, lane 5).
Cvt9 and Its P. pastoris Homologue, Gsa9, Are both Required for Peroxisome Degradation

We identified GSA9 as an essential component for the glucose-stimulated peroxisome degradation pathway in P. pastoris. Sequence analysis revealed GSA9 to be a structural and functional homologue of CVT9. Both proteins contain a predicted coiled coil domain. However, although many proteins encoded by the S. cerevisiae genome share common residues in the coiled coil domain, only Cvt9 shows extensive homology with its P. pastoris counterpart over the entire sequence. More specifically, a BLAST search with the NH2-terminal and COOH-terminal Gsa9 domains (amino acids 1–810 and 1028–1316, respectively) flanking its predicted coiled coil region was performed against all S. cerevisiae proteins (http://genome-www2.stanford.edu/cgi-bin/SGD/nph-blast2sgd). The BLAST results identified the corresponding NH2-terminal and COOH-terminal domains flanking the coiled coil region of Cvt9 as the only sequences with statistically significant similarity to Gsa9 (P value, 3.9 × 10⁻¹⁹ and 3.9 × 10⁻¹⁷, respectively). This sequence analysis indicates that Cvt9 is the sole homologue of Gsa9 in S. cerevisiae and does not represent one member in a family of related proteins.

To determine if Cvt9 was also required for the specific degradation of peroxisomes in S. cerevisiae, we examined the degradation of Fox3, the peroxisomal thiolase enzyme, following a previously described method for examining pexophagy in S. cerevisiae (Hutchins et al., 1999). Peroxisome proliferation was induced in cvt9Δ and wild-type cells by growth in oleic acid medium. Upon shift to SD-N medium to induce pexophagy, peroxisome degradation was examined by monitoring cellular Fox3 levels over the indicated time course (Fig. 6 A). In wild-type cells, Fox3 levels decreased significantly over the time course, whereas they remained constant in cvt9Δ cells. This finding suggests that Cvt9 is required for the specific, vacuolar delivery of not only prAPI via the Cvt pathway, but also peroxisomes via the pexophagy pathway in S. cerevisiae.

The P. pastoris gsa9 mutant was further characterized to better understand the cellular function of Gsa9. Peroxisomes and the expression of resident enzymes, such as AOX, were induced in methanol-containing medium. The peroxisomes were then degraded upon shift to glucose-containing medium. After a 6-h incubation in glucose adaptation conditions, AOX activity was measured (Fig. 6 B). Wild-type GS115 cells displayed significant loss of AOX activity, indicating that excess peroxisomes were degraded via pexophagy. As a negative control, gsa7 cells were analyzed. GSA7 is the P. pastoris homologue of APG7, an essential component for pexophagy as well as the Cvt pathway and autophagy (Yuan et al., 1999). Both gsa7 and gsa9 cells retained substantial AOX activity upon glucose adaptation, confirming the roles of these proteins in peroxisome degradation.

As we have noted, Cvt9 does not play an integral role in starvation-induced autophagy (Fig. 1). If Gsa9 functions in an analogous manner, then gsa9 mutants should also be competent for autophagic degradation. Autophagy accounts for the majority of the cellular degradative capacity during starvation conditions (for review see Kim and Klionsky, 2000). Thus, the degree of autophagy can be
measured in terms of total protein turnover under nitrogen starvation conditions. Wild-type, gsa7, and gsa9 cells were labeled with [14C]valine, followed by a nonradioactive chase reaction in nitrogen starvation medium. The production of TCA-soluble radioactivity was measured over the course of 2–24 h. The autophagy-defective gsa7 strain exhibited a low rate of protein turnover, whereas the relative protein degradation rate in gsa9 cells appeared similar to the wild-type, suggesting that gsa9 is not defective in autophagy (Fig. 6 C).

The process of micropexophagy can be categorized into distinct stages (Sakai et al., 1998). During the early steps of glucose adaptation, peroxisomes associate with the vacuolar membrane. Arm-like extensions of the vacuole initiate the peroxisome engulfment process. In the middle stage(s), vacuolar extensions become more pronounced, ultimately surrounding the peroxisomes. This is followed by the late stage(s), which involves a homotypic fusion event to fully enclose the peroxisomes inside the vacuole. Subsequent breakdown of the peroxisome-containing inner vacuolar vesicle triggers the eventual degradation of the peroxisomes. To determine the stage at which Gsa9 is required during micropexophagy, we examined wild-type, gsa9Δ, and gsa9ΔΔ strains by electron microscopy (Fig. 6 D). In wild-type cells, peroxisomes could be occasionally detected undergoing degradation in the vacuolar lumen. However, both gsa9-2 and gsa9Δ cells exhibited a micropexophagy defect at a middle/late stage in which vacuolar extensions only partially surround the peroxisome but do not complete the engulfment process.

Gsa9 Is Recruited to the Vacular Membrane during Peroxisome Uptake

To further characterize the role of Gsa9 in micropexophagy, we examined its localization by fluorescence microscopy using a functional GFP/HA-Gsa9 fusion protein. This construct was able to complement the gsa9ΔΔ phenotype (data not shown). In cells that have not been subjected to glucose adaptation conditions, GFP/HA-Gsa9 appeared localized to one or two structures adjacent to the vacuole (data not shown). In addition, a weak GFP/HA-Gsa9 signal was detected coincident with the FM 4-64–labeled vacuoles. The punctate pattern of GFP/HA-Gsa9 appeared nearly identical to the localization pattern of GFP/Cvt9, although we could not detect GFPCvt9 on the vacuole (Fig. 4 A). When cells expressing GFP/HA-Gsa9 were grown in methanol medium to proliferate peroxisomes and then subjected to adaptation in glucose medium, the GFP/HA-Gsa9 signal localized predominately to the vacuolar membrane (Fig. 7, top). To compare GFP/HA-Gsa9 localization to that of peroxisomes, we constructed a blue fluorescent protein (BFP) tagged with the serine-lysine-leucine COOH-terminal peroxisomal targeting signal (BFP-SKL; Fig. 7, middle). In glucose adaptation conditions, the intensity of GFP/HA-Gsa9 appeared strongest at the vacuolar regions that surrounded the peroxisomes during the engulfment process (Fig. 7, top). GFP/HA-Gsa9 also formed punctate structures that appeared continuous with its vacuolar membrane labeling pattern. The distribution of GFP/HA-Gsa9 before and after glucose adaptation suggests that Gsa9 localizes to regions of the vacuolar membrane that contact peroxisomes during micropexophagy.

Discussion

Autophagy and the Cvt pathway are overlapping processes that share most components. However, the Cvt pathway operates in a biosynthetic capacity during vegetative conditions, whereas autophagy is primarily a degradative process that is induced by starvation. Both pathways involve dynamic membrane rearrangements to deliver cytosolic contents to the vacuole. Morphological studies suggest that the two pathways normally operate exclusively of one another. The regulatory mechanisms and structural differences that control the conversion between the Cvt pathway and autophagy are not well understood. To gain further insight into the factors that are distinct between these pathways, we have examined a protein component, Cvt9, and its P. pastoris homologue, Gsa9, which appear to be specific to the Cvt pathway and micropexophagy, respectively.

Cvt9 Forms a Protein Complex Comprised of Cvt/Apg Phosphoproteins

Cvt9 exists primarily as a peripherally bound membrane protein (Fig. 3, B and C). Coimmunoprecipitation results demonstrate that it forms homodimers or even higher order homooligomers (Fig. 3 A), potentially through a coiled coil assembly mechanism, as indicated by secondary structural analysis. Consistent with our recent two-hybrid results (Kamada et al., 2000), we have demonstrated by coimmunoprecipitation analysis that Cvt9 directly interacts with the serine/threonine kinase Apg1 (Fig. 5), a component essential for both Cvt transport and autophagy. The association with Apg1 places Cvt9 as a member of a larger putative protein complex comprised of other components of the Cvt and autophagy pathways including Apg1, Apg13, Apg17, and Vac8 (Kamada et al., 2000; Scott et al., 2000). However, it remains to be determined if these Cvt/Apg components assemble into one protein complex or whether they represent separate, discrete interactions. Recent studies have demonstrated stable physical interactions between Apg1 and Apg13, Apg1 and Apg17, and Vac8 and Apg13 (Kamada et al., 2000; Scott et al., 2000). Apg13, together with Apg1, represent the components in the putative protein complex that are required for both Cvt transport and autophagy (Scott et al., 1996, 2000). In contrast, Apg17 is required for autophagy but not the Cvt pathway (Kamada et al., 2000), whereas Vac8 does not appear to be essential for autophagy but is required for the Cvt pathway (Scott et al., 2000). Similar to the vac8 phenotype, the cvt9 mutant also displays the hallmarks of a functional autophagy pathway, including viability under nutrient deprivation, the ability to transport bulk cytosol to the vacuole, and the accumulation of autophagic bodies when vesicle break down is pharmacologically inhibited (Fig. 1).

The differential effects that Cvt9, Apg1, Apg13, Apg17, and Vac8 exert on the Cvt and autophagy pathways suggest that these components may serve to regulate the conversion between Cvt transport and autophagy. Apg1,
Apg13, Cvt9, and Vac8 are all phosphoproteins, and the phosphorylation state and affinity of Apg13 to Apg1 are regulated by nutrient conditions through the Tor kinase cascade (Matsuura et al., 1997; Kamada et al., 2000; Scott et al., 2000). Taken together, these findings suggest that these regulatory proteins may serve as a junction at which upstream signals based on nutrient availability are transduced to effect the downstream formation of either Cvt vesicles or autophagosomes.

The Cvt9 Protein Complex Localizes to a Novel Perivacuolar Compartment

The subcellular distribution of GFP-Cvt9 supports an increasingly familiar localization pattern among Cvt and autophagy components (Kim et al., 1999; Noda et al., 2000). Specifically, an intense concentration of the GFP-Cvt9 fusion protein appears at a punctate, perivacuolar structure (Fig. 4 A). This localization pattern corresponds to a subcellular density profile distinct from other known membrane compartments, including ER, Golgi, and the Pep12-localized endosome (Fig. 4 B and C). Given that Cvt9 associates with Apg1, which in turn interacts with additional components essential for autophagy, the Cvt pathway, and pexophagy, the putative Cvt9 protein complex may identify, at the molecular level, a novel membrane compartment critical for these modes of subcellular trafficking.

Mechanistic Similarities between the Cvt/Autophagy Pathways and Micropexophagy

In P. pastoris, the specific degradation of excess peroxisomes during glucose adaptation entails the sequestration of peroxisomes by the vacuole in the process of micropexophagy. The mechanistic details of how a Cvt vesicle or an autophagosome forms to sequester cargo appear morphologically similar to the vacuole-mediated engulfment of peroxisomes. In each case, the sequestration of cargo by a membrane source results in the formation of a double-membrane structure. In the case of micropexophagy, the engulfing membrane is the vacuole itself whereas the membrane source in the formation of Cvt vesicles and autophagosomes remains to be determined.

We have identified Gsa9 as the P. pastoris homologue of Cvt9. The functional analysis of Gsa9 corroborates and may provide new insights into the role of Cvt9 in S. cerevisiae. Neither Gsa9 nor Cvt9 is required for autophagy, whereas both are essential for peroxisome degradation (Figs. 1 and 6). The degradation of peroxisomes in S. cerevisiae may occur by micropexophagy (Chiang et al., 1996). In P. pastoris, the uptake of peroxisomes by micropexophagy is easily detected and thus the morphological consequence of a gsa9 mutation can be readily assessed. Electron micrographs reveal that the arrest in micropexophagy occurs at a middle sequestration stage in the gsa9 mutant. That is, the vacuole(s) only partially surrounds the peroxisomes but cannot complete the engulfment process (Fig. 6 D).

The prAPI phenotype in cvt9 supports a similar defect in the Cvt pathway. To characterize the direct effect of Cvt9 inactivation, we constructed a Cvt9 temperature-degron fusion protein (Cvt9td), a novel temperature-conditional form of Cvt9 (Fig. 2 B). At nonpermissive temperature, prAPI in the cvt9td strain remained protease accessible (Fig. 2 C). In addition, prAPI was able to float through a step gradient in a detergent-dependent manner, suggesting that it associated with a floatable membrane (Fig. 2 D). Therefore, the cvt9 defect in the Cvt pathway may be at a middle-to-late stage in Cvt vesicle formation, analogous to the gsa9 mutant phenotype for micropexophagy.

Localization of Gsa9 Versus Cvt9

A key difference between Gsa9 and Cvt9 is their subcellular localization patterns. Before the induction of micropexophagy, Gsa9 appears to be localized to both a punctate structure in the perivacuolar region and diffusely distributed on the vacuole (data not shown). However, upon glucose adaptation, a population of Gsa9 strongly localizes to regions of the vacuolar membrane that are in contact with peroxisomes in the process of sequestration into the vacuole (Fig. 7). In contrast, the localization of Cvt9 to the perivacuolar compartment appears constant regardless of nutrient status. If Cvt9 functions analogously to Gsa9 in the Cvt pathway of S. cerevisiae, then it may directly participate in the engulfing of prAPI-containing Cvt complexes to form the double-membrane Cvt vesicles. Furthermore, if Cvt9 acts at the site of cargo sequestration in an analogous manner to Gsa9 at the vacuole during peroxisome engulfment, then the Cvt9 localization pattern would identify the perivacuolar compartment as the donor membrane source for Cvt vesicles. Additional studies will be required to test this assertion.

Potential Function of Gsa9 and Cvt9

Given that the mechanisms of the Cvt pathway and pexophagy are quite similar to autophagy (for review see Kim and Klionsky, 2000), why is autophagy not affected in the cvt9 and gsa9 mutants? The function of Cvt9 and Gsa9 may be required for the recognition of specific cargo, such as prAPI and peroxisomes, but not for the sequestration of nonspecific bulk cytosol by autophagy. The observation that Gsa9 appears to be concentrated in a region of the vacuolar membrane that contacts the peroxisome during the engulfment process suggests that Gsa9 may function in the recognition of peroxisomes. Without Gsa9, the proper associations between the peroxosomal and vacuolar membranes may not exist, resulting in a checkpoint stop in the engulfment process. The role of Gsa9 and Cvt9 as potential factors that provide cargo specificity is supported by the observation that deleting Cvt9 destabilizes the binding of prAPI-containing Cvt complexes to the membrane (Fig. 2 A). In addition, the role of Cvt9 in prAPI specificity can also be examined in terms of the prAPI reversal phenotype. Under nitrogen starvation conditions, prAPI accumulation is only partially reversed in the cvt9Δ strain (Fig. 1 C). If a deletion in CVT9 did not affect the selective nature of prAPI import, then one would predict that the entire population of prAPI would enter the vacuole via autophagy. The near complete reversal of prAPI under starvation conditions is observed in some API propeptide mutants that retain the ability to bind to the membrane (Scott et al., 1997). In contrast, the import of the bulk cytosolic marker Pho8Δ360 via autophagy plateaus at ~30%. Therefore, the loss of cargo selectivity in the cvt9Δ strain may result in the recognition of prAPI as another nonspe-
cific molecule during autophagy. Further analysis of Cvt9 and the proteins that associate with it will provide additional information about the conversion between the Cvt pathway and autophagy as well as the mechanism that modulates specificity in the Cvt pathway and pexophagy.

We thank Maria Hutchins for advice and helpful discussions on pexophagy; Kimberly Eggerton, Andrew Bevan, and Denny Player for technical assistance; and Wei-Fang Huang, Chao-Wen Wang, and Drs. Sarah Teter and Hagai Abeliovich for critical reading of the manuscript. We also thank Dr. Jurgen Dohme for supplying the plasmids used in constructing the cytoplasmic mutant and Dr. Dennis Thiele for supplying the vectors containing the yeast CUP1 inducible copper promoter.

This work was supported by Public Health Service grant GM53396 from the National Institutes of Health to D.J. Klionsky. Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan to Y. Ohsumi, a grant from The Norwegian Cancer Society to P.E. Stromhaug, and National Science Foundation grant MCB-9817002 to W.A. Dunn, Jr.

Submitted: 23 October 2000
Revised: 30 January 2001
Accepted: 20 February 2001

References


