Isolation and Characterization of Yeast Mutants in the Cytoplasm to Vacuole Protein Targeting Pathway

Tanya M. Harding, Kevin A. Morano, Sidney V. Scott, and Daniel J. Klionsky

Section of Microbiology, University of California, Davis, California 95616

Abstract. In *Saccharomyces cerevisiae* the vacuolar protein aminopeptidase I (API) is localized to the vacuole independent of the secretory pathway. The alternate targeting mechanism used by this protein has not been characterized. API is synthesized as a 61-kD soluble cytosolic precursor. Upon delivery to the vacuole, the amino-terminal propeptide is removed by proteinase B (PrB) to yield the mature 50-kD hydrolase. We exploited this delivery-dependent maturation event in a mutant screen to identify genes whose products are involved in API targeting. Using antiserum to the API propeptide, we isolated mutants that accumulate precursor API. These mutants, designated *cvt,* fall into eight complementation groups, five of which define novel genes. These five complementation groups exhibit a specific defect in maturation of API, but do not have a significant effect on vacuolar protein targeting through the secretory pathway. Localization studies show that precursor API accumulates outside of the vacuole in all five groups, indicating that they are blocked in API targeting and/or translocation. Future analysis of these gene products will provide information about the subcellular components involved in this alternate mechanism of vacuolar protein localization.

SUBCELLULAR compartmentalization is critical to eukaryotic cellular physiology. Accordingly, eukaryotes have evolved multiple mechanisms to deliver proteins to the various membrane bound subcellular compartments found in these cells. Among these organelles in the yeast *Saccharomyces cerevisiae*, the vacuole is involved in numerous cellular processes; the various functions of the vacuole rely on the specific and efficient delivery of its constituent resident proteins (Klionsky et al., 1990). Until recently it was thought that all proteins reach this organelle through a portion of the secretory pathway, transiting from the ER to the Golgi complex before being sorted into the vacuolar delivery pathway. An implicit assumption in this thought is that the ER is the only organelle in this system that has the capacity to translocate proteins across a lipid bilayer. However, it has recently been shown that at least two proteins are delivered to the vacuole by a post-translational mechanism that is independent of the secretory pathway (Yoshihisa and Anraku, 1990; Klionsky et al., 1992). The details of this alternate cytoplasm to vacuole targeting pathway have not been elucidated.

Studies on various organelles in yeast have revealed that the ability to transport proteins across an organellar membrane is the rule rather than the exception. In addition to the ER and vacuole, proteins are transported directly across the membranes of the mitochondria and peroxisome as well as the plasma membrane (Glick and Schatz, 1991; Aitchison et al., 1992; Michaelis, 1993). The Golgi complex is the principal subcellular compartment that appears to rely exclusively on vesicular transport for the delivery of its resident proteins. Among the membranes that are capable of direct protein import, the ER has been extensively characterized (reviewed in Pryer et al., 1992). Similarly, the protein import machinery in the mitochondrial inner and outer membranes has been the subject of substantial research (reviewed in Hannavy et al., 1993). In contrast, other mechanisms, and in particular the secretory pathway-independent routes to the vacuole, are less well understood.

The vacuole is unique in that proteins are known to enter this organelle by four different mechanisms (Fig. 1). (*a*) Most resident proteins are targeted to the vacuole via the secretory pathway (reviewed in Klionsky et al., 1990). This includes the well characterized vacuolar proteins carboxypeptidase Y (CPY)[1] and proteinase A (PrA) (Stevens et al., 1982; Klionsky et al., 1988). (*b*) Extracellular proteins destined for degradation enter the vacuole through endocytosis (Riezman, 1985; Raths et al., 1993); for example,

Address correspondence to Daniel J. Klionsky, Section of Microbiology, Hutchison Hall, University of California, Davis, CA 95616. Tel.: (916) 752-0277. FAX: (916) 752-9014. e-mail: djklionsky@ucdavis.edu

1. *Abbreviations used in this paper:* API, aminopeptidase I; CPY, carboxypeptidase Y; cvt, cytoplasm to vacuole targeting; EMS, ethyl methane sulfonate; PGK, phosphoglycerate kinase; PrA, proteinase A; PrB, proteinase B; vps, vacuolar protein sorting; YPD, 1% yeast extract, 2% peptone, and 2% dextrose media.

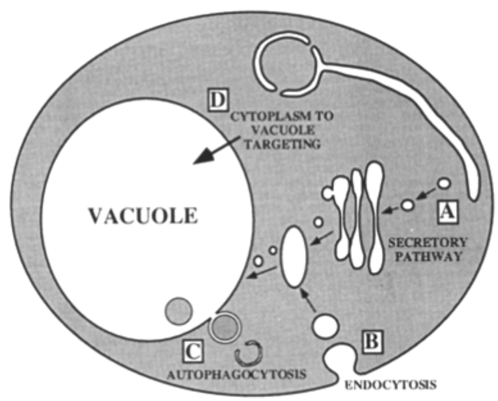

Figure 1. Four routes for protein delivery into the yeast vacuole. (*A*) Newly synthesized vacuolar proteins that contain an amino-terminal or internal signal sequence are cotranslationally translocated into the ER and transit, via vesicular intermediates, to the *trans*-Golgi network, where they are sorted away from secreted proteins. Targeting determinants within the protein are necessary for this sorting event and for subsequent targeting to the vacuole. (*B*) Endocytosis provides a pathway to the vacuole for extracellular proteins that are destined to be degraded. This pathway intersects the secretory pathway (*A*) at the prevacuolar or endosomal compartment (Davis et al., 1993; Singer-Kruger et al., 1993; Vida et al., 1993). (*C*) Autophagy is responsible for the vacuolar degradation of cytosolic components. This process occurs through the formation of a double-membrane vesicle containing cytosol. The outer layer of the autophagic vesicle fuses with the vacuolar membrane to release an autophagic body into the vacuolar lumen for subsequent degradation (Baba et al., 1994). (*D*) Newly synthesized API enters the vacuole posttranslationally, using a direct cytoplasm to vacuole targeting mechanism.

mating pheromones and their receptors are internalized and delivered to the vacuole via an endosomal intermediate (Chvatchko et al., 1986; Singer and Riezman, 1990; Davis et al., 1993). (*c*) Under starvation conditions, cytoplasmic proteins are taken into the vacuole by autophagocytosis (Takeshige et al., 1992; Baba et al., 1994; Thumm et al., 1994), presumably to allow recycling of critical metabolites. (*d*) Some resident proteins, in particular α-mannosidase and API, enter this organelle by an alternate mechanism directly from the cytoplasm. It is not known if these two proteins employ the same targeting mechanism for vacuolar localization.

API serves as a useful marker protein to examine this alternate vacuolar targeting pathway (Klionsky et al., 1992). It is synthesized as a soluble cytosolic protein containing an amino-terminal propeptide. Upon delivery to the vacuole, this propeptide is proteolytically removed to generate the lower molecular mass, mature form of the enzyme. The signal used by API that allows it to be directed to the vacuole and sorted away from other cytoplasmic proteins has not been characterized. It is also not known if there are specific receptor proteins on the vacuolar membrane that recognize API and direct its interaction with the import machinery. Overproduction of API results in accumulation of the precursor form of this hydrolase, suggesting that one or more of the component(s) required for import is saturable. Because API enters the vacuole posttranslationally, it may also need to interact with cytosolic chaperones (Hendrick and Hartl, 1993) that maintain a translocation competent conformation. Overproduction of API would result in the titration of these or other limiting proteins, such as a membrane-bound receptor, and cause the observed precursor accumulation phenotype.

We decided to employ a genetic approach to elucidate the alternate targeting pathway used by API. The delivery-dependent maturation event was used to identify yeast mutants that are defective in this pathway. We have isolated a collection of mutants that accumulate the unprocessed precursor form of API. Of these, two complementation groups are allelic to previously identified *vps* class B mutants (Banta et al., 1988; Raymond et al., 1992*a*), one is allelic to *PRB1*, the gene encoding proteinase B, and the remaining five show no allelism to tested mutants. These five are phenotypically distinct from *vps* mutants, showing no secretion of CPY or significant defect in its maturation. Characterization of the effects of these mutants on vacuolar protein biogenesis indicates that the five unique complementation groups are specifically defective in targeting API to the yeast vacuole. Analysis of these mutants will allow the identification of components involved in cytoplasm to vacuole protein targeting.

Materials and Methods

Strains and Media

The yeast strains used in this study are listed in Table I. All yeast strains were grown in YPD (1% yeast extract, 2% Bacto peptone, and 2% dextrose) for Western analysis, or in synthetic minimal medium supplemented with the appropriate amino acids (Guthrie and Fink, 1991) for radiolabeling and immunoprecipitations. Yeast were typically grown at 30°C and harvested at an OD_{600} of 0.8 to 1.2, unless otherwise noted. Lithium acetate yeast transformations, genetic crosses, diploid selection, tetrad dissection, and analysis, and complementation testing were carried out essentially as described (Guthrie and Fink, 1991).

Reagents

Zymolyase 20T was obtained from ICN Biomedicals (Irvine, CA). Expre^{35}S^{35}S protein labeling mix and autoradiographic film were from Dupont-NEN Research Products (Boston, MA). Glass beads (0.45–0.52 μm) were from Thomas Scientific (Sweetborough, NJ), acrylamide and proteinase K were from Boehringer Mannheim Biochemical Corp. (Indianapolis, IN), 0.45 μ BA-S nitrocellulose was from Schleicher and Schuell (Keene, NH), and Immobilon-P (polyvinylidene fluoride, PVDF) was from Millipore (Marlborough, MA). Antisera to CPY and PrA were described previously (Klionsky et al., 1988), as was antiserum to peptides within the mature region of API (Klionsky et al., 1992). Antisera to PrB and phosphoglycerate kinase (PGK) were generously provided by Charles Moehle and Elizabeth Jones (Moehle et al., 1989), and Jeremy Thorner (Baum et al., 1978), respectively. Autofluor was from National Diagnostics, Inc. (Manville, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Plasmids

pRN1 (centromeric *APEI*) and pRC1 (2μ *APEI*) were both previously described (Klionsky et al., 1992). The plasmid encoding *PRB1*, FP8 (Moehle et al., 1987), was supplied by Charles Moehle and Elizabeth Jones. This plasmid was restriction digested with BamHI and HindIII, and the ends were filled in with the Klenow fragment of DNA polymerase I. The resultant blunt-ended fragment was ligated into the SmaI site of plasmid pSEY8 (Emr et al., 1986) to construct plasmid pSEY8-*PRB1*.

Antibody Generation

To produce antiserum that specifically recognized the propeptide region of API, a peptide was synthesized (Multiple Peptide Systems, San Diego,

Table I. Yeast Strains Used in Mutant Generation and Complementation Testing

Strain	Genotype	Source
SEY6210	MATα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9	Robinson et al., 1988
SEY6211	MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9	Robinson et al., 1988
vps1-6, 8-35	derived from SEY6210 of SEY6211	Robinson et al., 1988
SEY2101 ΔPEP4	MATa leu2-3,112 ura3-52 ade2-101 suc2-Δ9 pep4::LEU2	Bankaitis et al., 1986
DYY101	SEY6210 Δape1::LEU2	Klionsky et al., 1992
SF838-10	MATα ade6 his4-519 ura3-52 leu2,3-112 pep4-3	Rothman and Stevens, 1986
SF838-90	MATa ade6 his4 leu2 ura3 pep4	Rothman and Stevens, 1986
SF838-9DR2L1	MATa his4-519 ura3-52 leu2,3-112 lys2 pep4-3	Rothman and Stevens, 1986
vps7, 36-39,41, 43-46	derived from SF838-9DR2L1, SF838-90, or SF838-10	Rothman and Stevens, 1986
AFM69-1A	MATα ura3-1 his3,11-15 leu2,3-112 sec7-4	Franzusoff and Schekman, 1989
ANY123	MATα ura3-52 his4-619 bet1	Newman et al., 1990
BJ993	MATα trp1 pep9-2	Rothman et al., 1989
BJ1014	MATα trp1 pep13-3	E. Jones
BJ1027	MATα trp1 pep2-4	E. Jones
BJ4000	MATα ade6 his1 trp1 leu2 ura3 pep7	E. Jones
BJ4786	MATα ura3-52 leu2 trp1 pep1-9	E. Jones
CTY1-1A	MATa ura3-52 Δhis3-200 lys2-801am sec14-1	Bankaitis et al., 1989
LW153	MATa ura3-52 ade vac1	Weisman et al., 1990
NY424	MATα ura3-52 sec21-1	Newman et al., 1990
NY425	MATα ura3-52 sec22-3	Newman et al., 1990
RSY27	MATα ura3-52 his4-593 sec59-1	R. Schekman
PBY408A	MATα ura sec11	Bohni et al., 1983
RDM50-94C	MATα ura 3-52 leu2,3-112 his4 sec62	Rothblatt et al., 1989
RSY12	MATα ura3-52 leu2,3-112 sec53-6	R. Schekman
SEY5018	MATα ura3-52 leu2,3-112 sec1-1	S. Emr
SEY5186	MATα ura3-52 leu2,3-112 suc2-Δ9 sec18-1	Klionsky et al., 1988
THY14	SEY6211 cvt1-1	This study
THY193	SEY6211 cvt2-1	This study
THY119	SEY6211 cvt3-1	This study
THY215	SEY6211 cvt4-2	This study
THY313	SEY6211 cvt5-1	This study
THY144	SEY6211 cvt6-1	This study
THY154	SEY6211 cvt7-1	This study
THY195	SEY6211 cvt8-1	This study

CA) that corresponded to amino acid residues 18–43 based on the deduced amino acid sequence of the *APE1* gene (Chang and Smith, 1989; Cueva et al., 1989). The peptide (NH_3-CWSNENEKKEKEENAIQNNK-SPEVTL) was coupled to keyhole limpet hemocyanin at the amino-terminal cysteine and used to immunize two male New Zealand White rabbits (0.7 mg per rabbit followed by multiple injections of 0.1 mg).

Isolation of Mutants Defective in API Maturation

Yeast cells (SEY6210 transformed with pRN1) were grown overnight in YPD, washed twice in 50 mM sodium phosphate, pH 6.5, and then resuspended in this buffer at ~10 OD_{600} units. Ethylmethane sulfonate (EMS) was added to a final concentration of 30 μl/ml, and the cells were incubated on a roller drum for 1 hour at 30°C. The cells were washed once with 5% (wt/vol) sodium thiosulfate to quench the EMS, washed twice with water, then spread onto YPD plates and grown 5 d at 23°C. This mutagenesis resulted in an ~50% decrease in viability.

Two related procedures were used for mutant isolation. In the first, colony hybridizations were made from the original spread plates using a modified technique based on Lyons and Nelson (1984). Yeast were mutagenized with EMS as described above and spread onto YPD plates at a density of 150–300 colonies per plate. After 5 d growth at 23°C, a nitrocellulose filter was overlaid onto the colonies and left in place 1 min. Each filter was then removed and placed colony side up onto a fresh YPD plate, followed by incubation at 23°C for 3 h. Filters were then placed on Whatman paper saturated with 67 mM potassium phosphate, pH 7.5, 20 mM EDTA, 20 mM DTT, and incubated 5 min at room temperature. The filters were then transferred to Whatman paper saturated with 5% SDS and incubated for 30 min at 37°C. Cell debris was washed off of the filters under running distilled water. Colonies accumulating precursor API were identified using the API propeptide-specific antiserum as described below.

In the second procedure, the mutagenized cells were spread onto YPD plates, grown 5 d at 23°C, and individual colonies patched onto fresh YPD plates and allowed to grow for another 2 d. Colonies were transferred into microcentrifuge tubes (~0.5 to 1.0 OD_{600} equivalents per colony) in batches of 15 colonies per tube, and resuspended in 100 μl of lysis buffer (50 mM sodium phosphate, 20 mM MES, pH 7.0, 1% SDS, 3 M urea, 1 mM sodium azide, and 2 mM PMSF). Acid washed glass beads were added to 60% of the sample volume, and the samples mixed on a vortex mixer for 1 min. Each sample was then heated 10 min at 65°C and 500 μl fresh lysis buffer was added. The sample was cleared of cell debris by centrifugation for 5 min at 12,500 *g*. A 250-μl aliquot of the resultant lysate was adsorbed onto a nitrocellulose membrane using a 96-well format dot-blot apparatus (GIBCO BRL, Gaithersburg, MD). The filters were probed using the API propeptide-specific antiserum as described below. Batches of colonies that showed strong reactivity to the API propeptide antiserum were reanalyzed by dot-blot to examine individual colonies.

Positive colonies identified by either procedure were further analyzed by Western blot. Fresh protein extracts were prepared by glass bead lysis and separated on 10% polyacrylamide–SDS gels (Laemmli, 1970). After electrophoresis, the proteins were transferred (60–90 min) to PVDF membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (Towbin et al., 1979) at 4°C, and the blots probed with antiserum to mature API.

Immunodetection

After the dot-blot procedure, nitrocellulose membranes were autoclaved for 10 min in distilled water. Both nitrocellulose and PVDF membranes were then blocked for at least 30 min in 100 mM Tris-HCl, 0.9% NaCl with 0.1% Tween-20 (TTBS) (Towbin et al., 1979) containing 5% nonfat milk.

For immunodetection, primary antiserum to PrB was used at a dilution ratio of 1:10,000, antisera to API and PrA at 1:15,000, antiserum to CPY at 1:30,000, and antiserum to PGK at 1:75,000 in TTBS with 0.5% nonfat milk, and incubated with the blot for 1–9 h. After incubation in primary antiserum, all blots were washed in TTBS three times and incubated with horseradish peroxidase-conjugated goat anti–rabbit secondary antiserum (Cappel Labs., Cochranville, PA) at a dilution of 1:30,000 for 30–45 min. After incubation with the secondary antiserum, the filters were washed three times in TTBS, and immunoreactive bands visualized using a chemiluminescent substrate (Gallagher, 1994). Primary antiserum incubations of longer than 2 h were carried out at 4°C. All other incubations were carried out at room temperature. Quantification of Western blots was performed using the BioImage analysis system (Millipore Corp., Bedford, MA).

Labeling and Immunoprecipitation

Procedures for preparation and labeling of yeast cells were essentially as described (Klionsky et al., 1992). Double immunoprecipitation experiments were as described, except that each protein A–sepharose pellet was washed only once with Tween-IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA), and protein A–sepharose-bound proteins were eluted at 77°C for 10 min rather than boiling for 4 min.

To examine secretion of CPY from whole cells, yeast were labeled as described (Klionsky et al., 1992) for 20 min in the presence of BSA (2.5 mg/ml). Nonradioactive chase was initiated by the addition of 1 mM methionine, 2 mM cysteine, and 0.2% yeast extract and continued for 60 min. Samples were then separated into cell and medium fractions by a 10-s centrifugation at 12,500 *g*, and each fraction precipitated in 10% TCA. CPY was immunoprecipitated from the resultant extracts as stated above, and analyzed by SDS-PAGE. Quantification was done with phosphorimage analysis using the Fuji FUJIX BAS1000 Bioimaging Analyzer (Fuji Medical Systems, Stamford, CT).

Microscopy

Vacuolar acidification was examined by quinacrine accumulation and fluorescence using modifications of published procedures (Guthrie and Fink, 1991; Morano and Klionsky, 1994). Cells were grown in YPD to an OD_{600} of 0.9–1.2, and 1.0 ml was harvested and washed once with uptake buffer (YPD buffered to pH 7.6 with 100 mM Hepes). The cell pellet was resuspended in 50 μl fresh uptake buffer, and quinacrine added to a final concentration of 400 μM. Cells were incubated at 30°C for 7 min, held on ice for 5 min, then washed three times with ice-cold wash buffer (10 mM Hepes, pH 7.6, 2% glucose). Cell pellets were resuspended in 100 μl wash buffer. A 5-μl aliquot was placed on a microscope slide, mixed with 5 μl of 1% low-melt agarose, and covered with a coverslip. Stained cells were photographed using Kodak X-125 black and white film within 1 h of quinacrine staining, using a Nikon Labophot-2 microscope equipped with direct interference contrast optics for Nomarski photomicrographs. Fluorescence filter cube BV-2B (excitation λ = 400–440 nm, emission λ = 480 nm) was used for imaging of quinacrine fluorescence.

Endocytosis was examined by accumulation of lucifer yellow as described (Riezman, 1985), and cellular fluorescence was imaged as detailed above.

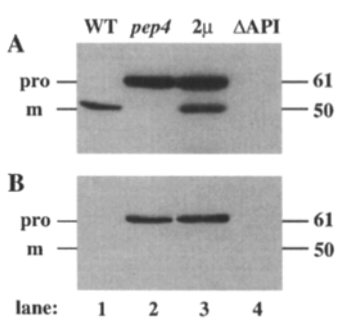

Figure 2. Precursor API is not detected in wild-type cells under steady-state conditions. Crude protein extracts were prepared from the following strains: SEY6210 transformed with pRN1 (WT), or with pRC1 (2μ), SEY2101 Δ*pep4* (*pep4*) and DYY101 (Δ*API*). Approximately 0.2 OD_{600} (lanes *1*, *2*, and *4*) or 0.05 OD_{600} (lane *3*) equivalents of cells of each extract were separated by SDS-PAGE on two identical 10% gels, transferred to PVDF membrane, and probed as described in Materials and Methods with (*A*) anti-mAPI antiserum, or (*B*) anti-proAPI antiserum. The positions of precursor and mature API are shown. The numbers at the right indicate the molecular mass in kD.

Cell Fractionation

Cells (20 OD_{600} units) were harvested at OD_{600} of 0.8–1.2, washed once in 10 mM Tris-SO_4, pH 9.4, 10 mM DTT, and resuspended in this buffer to an OD_{600} of 2.0. The cells were incubated for 15 min at 30°C with constant shaking (200–250 rpm), then harvested by centrifugation and resuspended in 1 M sorbitol, 20 mM Pipes, pH 6.8 (spheroplasting buffer), to an OD_{600} of ~1.0. Zymolyase 20T (0.2 mg) was added and the sample mixed gently by inversion. Spheroplasting was carried out at 30°C for no more than 30 min, with gentle mixing every 4–5 min. This procedure routinely yielded 85–95% spheroplasts in 15 min.

Spheroplasts were harvested by centrifugation at 3,000 *g* for 3 min, resuspended in spheroplasting buffer, and transferred to microcentrifuge tubes. The spheroplasts were harvested again at 3,000 *g* for 3 min, then gently resuspended in 1 ml of lysis buffer (150 mM sorbitol, 20 mM Pipes, pH 6.8), and incubated on ice for 5 min, inverting once at 2.5 min. A 400-μl aliquot was then removed (total sample) and precipitated with acetone. The remaining 600-μl aliquot was centrifuged at 12,500 *g* for 3 min, and the supernatant fraction removed and precipitated with acetone. The pellet was washed once with another 500 μl of lysis buffer, then precipitated. All precipitated samples were dried and resuspended in sample buffer (125 mM Tris-HCl, pH 6.8, 0.4% SDS, 1% glycerol, 5% β-mercaptoethanol) (Laemmli, 1970) at 50 μl per initial OD_{600} unit of cells, and analyzed by SDS-PAGE.

To examine the protease sensitivity of the accumulated proAPI, spheroplasts were prepared and osmotically lysed as above. 2 OD_{600} equivalents of lysed spheroplasts were then subjected either to a mock protease treatment or treatment with 50 μg/ml proteinase K for 30 min on ice in the presence or absence of 0.2% Triton X-100. Each sample was then precipitated, resuspended in sample buffer, and analyzed by SDS-PAGE as above.

Results

A Genetic Screen to Analyze Cytoplasm to Vacuole Targeting

To learn more about the novel localization mechanism utilized by API we decided to isolate yeast mutants that were defective for API targeting but that did not affect secretory pathway transport. The unique targeting mechanism of API, however, was problematic for mutant selection. Yeast mutants in the secretory pathway have been identified previously using selections that relied on secretion or sequestration of essential proteins (Bankaitis et al., 1986;

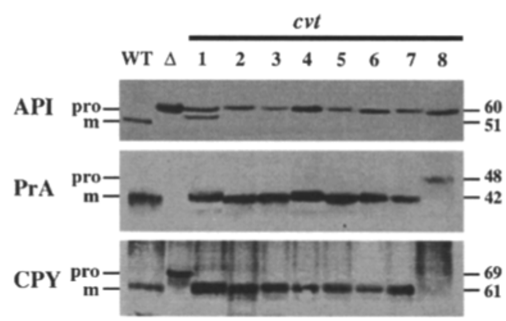

Figure 3. The *cvt* mutants accumulate precursor API. Cell extracts were prepared as described in Materials and Methods. Identical amounts (0.2 OD_{600} equivalents of cells) were separated on three 8% SDS-PAGE gels. Proteins were transferred to PVDF membrane and probed with anti-mAPI, anti-PrA, and anti-CPY antisera as stated in Materials and Methods. The position of precursor and mature forms of API, PrA, and CPY are given. Wild-type (SEY6210; WT) and SEY2101 Δ*pep4* (Δ) contain the centromeric plasmid pRN1. Lanes *3–10* contain the *cvt* strains listed in Table I. Numbers at the right indicate the molecular mass in kD.

Rothman and Stevens, 1986; Deshaies and Schekman, 1987). Because API does not transit through the secretory pathway, it is not possible to select for mutants that secrete this hydrolase. Also, the long half-time of import of API into the vacuole made selections based on sequestration of an essential cytosolic enzyme via this pathway impossible. For these reasons, we decided to carry out a direct screen for yeast mutants that accumulate the precursor form of API.

The rationale behind this approach was based on the observation that, even though API has a half-time of processing of 30–45 min, under steady-state conditions we do not usually detect significant amounts of precursor API in wild-type cells (Fig. 2, lane *1*). We could therefore use proAPI accumulation as a marker for defective targeting. To identify cells that accumulate proAPI, we produced polyclonal antiserum specific to the propeptide region of API. The reactivity of this antiserum (henceforth referred to as anti-proAPI) was compared to that of an antiserum directed against sequences in the mature portion of API (anti-mAPI) (Klionsky et al., 1992) by Western blot (Fig. 2). In wild-type cells harboring a centromeric plasmid encoding the *APE1* gene, API is seen as a single band of 50 kD when probed with anti-mAPI. An identical blot, probed with anti-proAPI, shows no reactivity (Fig. 2, compare lanes *1* in *A* and *B*). When equivalent samples are examined from cells in which the chromosomal copy of *PEP4* is disrupted, both antisera detect a single band at 61 kD, corresponding to the precursor form of API. Upon overproduction of API from a 2μ plasmid (pRC1), much of the newly synthesized precursor API protein becomes incompetent for maturation (Klionsky et al., 1992; Fig. 2 *A*, lane *3*). Only the accumulated precursor is specifically detected by anti-proAPI antisera. There is no cross-reactivity to other proteins in yeast, as can be seen in extracts from the *APE1* disruption strain DYY101 (Fig. 2 *B*, lane *4*).

Isolation of Mutants That Specifically Accumulate proAPI

The fact that wild-type cells do not accumulate significant amounts of precursor API under steady-state conditions, coupled with the specificity of the anti-proAPI antisera, allowed us to use an immunoblot-based screen to identify mutants in cytoplasm to vacuole targeting of API. To eliminate recovery of mutants in the gene encoding API, we transformed the wild-type strain SEY6210 with pRN1, a centromeric plasmid carrying the *APE1* gene. The transformed wild-type strain was mutagenized using EMS, plated onto nonselective media, and colonies examined immunologically for accumulation of proAPI. Two methods were employed for preliminary immunological identification of mutants: colony hybridization and dot blots (see Materials and Methods). After preliminary identification, extracts prepared from putative mutants were resolved by SDS-PAGE, transferred to PVDF, and probed with anti-mAPI antiserum to confirm the precursor accumulation phenotype. Out of 5,300 mutagenized yeast colonies, 16 indepen-

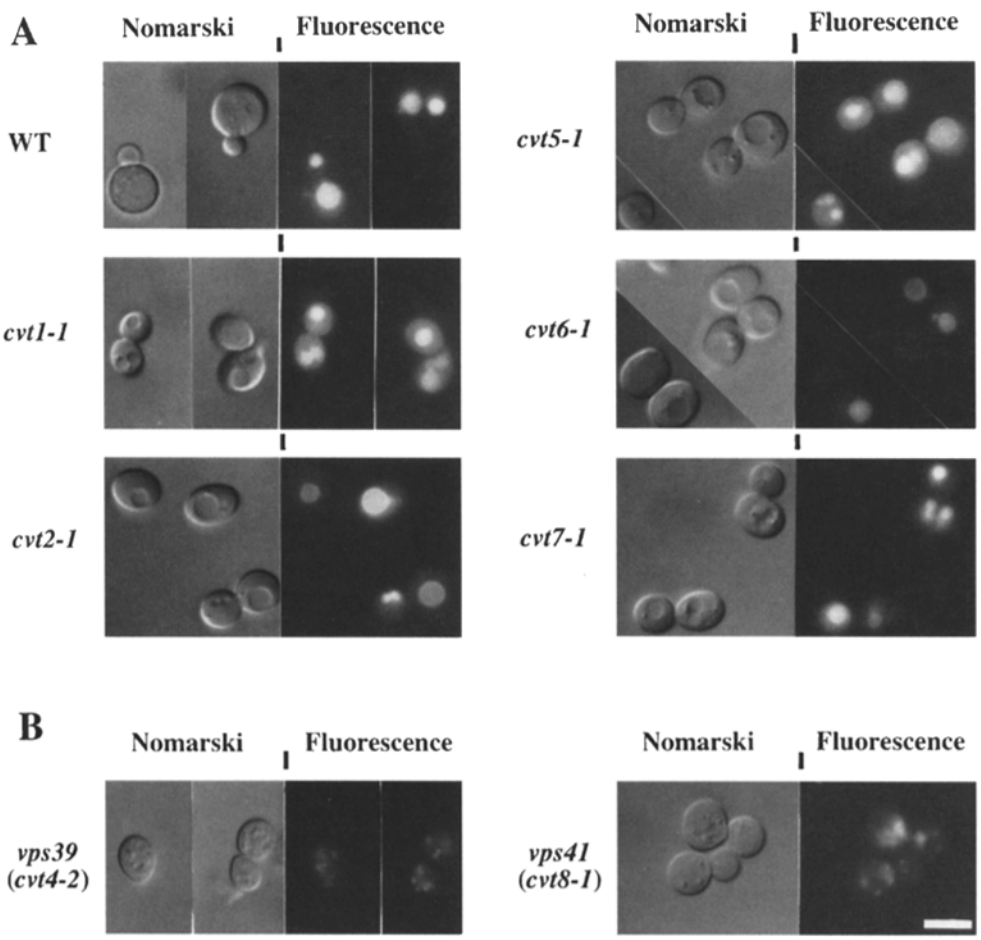

Figure 4. Vacuolar morphology and acidification in *cvt* mutants. Yeast strains were grown in YPD and loaded with quinacrine as described in Materials and Methods. (*A*) WT SEY6210, THY14 (*cvt1-1*), THY193 (*cvt2-1*), THY313 (*cvt5-1*), THY144 (*cvt6-1*), and THY154 (*cvt7-1*). The *cvt3* mutant appears the same as the wild-type strain (data not shown). (*B*) THY215 (*vps39/cvt4-2*) and THY195 (*vps41/cvt8-1*); *cvt4-1* has an identical phenotype to *cvt4-2* (data not shown). Bar, 5 μm.

dent mutants were isolated that consistently accumulated significant amounts of proAPI (Fig. 3). Henceforth, we refer to these mutants as *cvt*, for cytoplasm to vacuole targeting defective mutants. The mutants were determined to be unlinked to the plasmid pRN1 (data not shown), and each mutant was back-crossed to the isogenic wild-type strain (SEY6211) a minimum of three times to remove any extraneous mutations caused by EMS treatment. After three backcrosses, all 16 mutants segregated 2:2 for the accumulation of precursor API, indicating that they are all caused by single nuclear mutations (data not shown). These 16 mutants fell into eight complementation groups with the following allele distribution: *cvt1* has six identified alleles; *cvt2*, *cvt3*, and *cvt4* have two alleles each; and *cvt5* through *cvt8* have one allele each. The fact that four of our complementation groups contain only one allele suggests that our screen did not identify all of the genes involved in API targeting and delivery. A representative allele from each complementation group was chosen for subsequent analysis. None of the strains show differential accumulation of precursor or mature API at elevated (37°C) or reduced (23°C) temperatures (data not shown). However, it should be noted that all alleles of *cvt4* and *cvt8* exhibit reduced growth rates at 30°C and above when compared to the isogenic parental strain; the remaining *cvt* strains show no apparent growth defects.

The cvt Mutants Accumulate Precursor API

The processing of API in protein extracts from representative alleles of each *cvt* complementation group is shown in Fig. 3. With the exception of *cvt1*, all the mutants accumulate essentially 100% proAPI. The *cvt1* allele accumulates one or more intermediate-sized bands under normal growth conditions. We believe these intermediates are the result of *PEP4*-independent removal of portions of the propeptide, as equivalent bands are sometimes observed in strains with a disruption of the *PEP4* gene (data not shown).

As maturation of API is dependent on delivery of both proAPI and the PrA and PrB endoproteases to the vacuole, severe defects in the secretory pathway that affect delivery of these latter hydrolases would lead to accumulation of proAPI. Though such mutations could be identified using this screen, they would also cause detectable defects in the targeting and maturation of other vacuolar hydrolases that are transported via the secretory pathway. To determine if any of the *cvt* mutants displayed pleiotropic effects on vacuolar protein targeting, we examined the maturation state of other vacuolar hydrolases, as shown in Fig. 3. Based on this immunoblot analysis, most of the mutants isolated (*cvt2, cvt3, cvt5, cvt6,* and *cvt7*) appear to be specifically defective in API maturation. The *cvt8* mutant is notable in that it severely affects the maturation of the two secretory pathway targeted proteins CPY and PrA, accumulating proPrA as well as low levels of proCPY. Neither of these proteases is matured to a detectable level in *cvt8*. Alleles of *cvt1* and *cvt4* accumulate CPY at what appears to be its normal mature molecular mass of 61 kD at steady-state. PrA found in these strains, however, migrates at a slightly higher molecular mass (43 kD) than that observed for the wild-type strain (42 kD). It has previously been shown that PrA maturation is in part dependent on PrB function; in strains lacking PrB, PrA accumulates in the vacuole as an active, 43-kD form (Hirsch et al., 1992).

Morphology and Acidification of Vacuoles in cvt Mutants

A large group of mutants (*vps*) has been isolated that specifically disrupt vacuolar targeting through the secretory pathway (reviewed in Raymond et al., 1992*b*; Stack and Emr, 1993). Many of the *vps* mutants show gross alterations in vacuolar morphology. Maturation of API is partially blocked in several *vps* mutants, especially those referred to as class C mutants, which lack an identifiable vacuole structure when examined by light microscopy (Klionsky et al., 1992; Raymond et al., 1992*a*). Recent studies also have shown that mutations in *VMA* genes, which encode subunits of the vacuolar-type ATPase, result in lack of vacuolar acidification and cause the accumulation of precursor forms of vacuolar proteins that transit through the secretory pathway (Klionsky et al., 1992; Morano and Klionsky, 1994). Preliminary studies have shown that maturation of API is also partially defective in yeast that lack a functional vacuolar ATPase (K. Morano and D. Klionsky, unpublished data). For morphological characterization of the *cvt* mutant strains, we examined both structure and acidification of the vacuole. Acidification can be examined directly using the vital fluorescent dye quinacrine; this acidotropic molecule has been shown to accumulate in acidified compartments and has been useful in morphological examination of the vacuole by fluorescence microscopy (Roberts et al., 1991).

The *cvt* mutants were grown to mid-log phase, stained with quinacrine, and then examined using fluorescence and Nomarski optics. In all strains, vacuoles or vacuole fragments were seen to stain with quinacrine, indicating that there is no defect in vacuolar acidification in any of the *cvt* mutants (Fig. 4). The *cvt* vacuolar morphology, however, fell into two distinct classes (Fig. 4, compare staining in *A* to that seen in *B*). Most of the mutants, including *cvt1, 2, 3, 5, 6,* and *7*, contained one large or a few medium-sized, round vacuoles that were essentially indistinguishable from those observed in wild-type cells. The second morphological class, containing all the alleles of *cvt4* and *cvt8* (*vps39* and *vps41*, respectively; see below), showed a pattern of small, distinct punctate structures staining in a diffuse manner with quinacrine (Fig. 4 *B*). These punctate structures were also detectable by Nomarski optics. No large, single vacuole was ever observed in the *cvt4* or *cvt8* cells. This punctate morphology and staining pattern is reminiscent of the *vps* class B mutants (Banta et al., 1988; Raymond et al., 1992*a*), which accumulate severely fragmented vacuoles, and exhibit pleiotropic defects in vacuolar protein targeting. Therefore, two lines of evidence indicated that *cvt4* and *8* were *vps* mutants: both caused detectable defects in the maturation of multiple vacuolar proteases, and both have altered vacuolar morphology.

Allelism of cvt Mutants with Other Mutants That Perturb the Vacuolar System

To avoid extensive examination of previously identified mutants, we crossed the representative alleles from each *cvt* group to known mutants (Table I). We examined mutants that have been shown to affect protein targeting, protein maturation, and maintenance of the vacuole itself, including representatives from the *sec, vps, vac,* and *pep* groups. Diploids were generated and examined for complementation of the API precursor accumulation defect by Western blot (data not shown). The only allelisms encountered were as follows: *cvt4* was unable to complement a strain defective in *vps39*, and *cvt8* was unable to complement a strain defective in *vps41*, both of which are class B *vps* mutants. This result is in agreement with our Western analysis and morphological observations (Figs. 3 and 4). Henceforth, we will refer to mutants in these complementation groups by their previously identified gene names.

Because *PRB1* encodes the protease directly responsible for the maturation of API, we expected to obtain mutants defective in this gene. To determine whether or not the *cvt* mutants were defective in *PRB1* production and/or function, we examined the processing kinetics of PrB. PrB is synthesized as a 76-kD preproprotein, which enters the secretory pathway cotranslationally at the ER and is subsequently sorted to the vacuole. It undergoes several modifications during transit, including glycosylation and both PrA-dependent and -independent proteolytic cleavages, ultimately yielding a mature protein of 31 kD in the vacuole (Mechler et al., 1988; Moehle et al., 1989). In PrA-deficient mutants, PrB is targeted correctly but not matured beyond its Golgi-processed form (40 kD) (Moehle et al., 1989) (Fig. 5). Cells were grown to mid-log phase, labeled with [^{35}S]methionine for 5 min, and then subjected to a nonradioactive chase. Samples were removed at the times indicated and immunoprecipitated with antiserum to PrB. PrB maturation and expression levels were essentially indistinguishable from wild-type in *cvt2, 3, 5, 6,* and *7* (Fig. 5 and data not shown). PrB in *cvt1-1* appears to be produced at approximately wild-type levels, but is both processed more slowly and is unstable in the cell at long chase points (Fig. 5, 90-min chase). The proAPI accumulation defect in *cvt1-1* could be complemented by transformation with a centromeric plasmid carrying *PRB1*, pSEY8-*PRB1*, indicating that the alleles in this complementation group are defective in the gene encoding proteinase B.

Finally, we examined the *cvt* mutants for defects in vacuole inheritance and endocytosis. None of the unique complementation groups exhibit a defect in vacuole inheritance, based on the presence of tubular inheritance structures and vacuoles in the buds as detected by staining with the vital dye FM4-64 (Vida and Emr, 1995; Lois Weisman, personal communication). Vacuolar accumulation of extracellular lucifer yellow was monitored as an assay for endocytosis (Riezman, 1985). None of the unique *cvt* mutants show a defect in endocytosis as judged by this technique (data not shown). Our allele of *vps39* (*cvt4*) shows only low levels of lucifer yellow staining in fragmented vesicles, similar to that observed with quinacrine (Fig. 4). Interestingly, the allele of *vps41* (*cvt8*) accumulates a small number of brightly fluorescent bodies just inside of the cell periphery after a 2-h incubation with lucifer yellow; at this time point the wild-type controls have brightly stained vacuoles, as do all of the other *cvt* mutants (*1, 2, 3, 5, 6,* and *7*). Finally, mutants in endocytosis do not affect API maturation (data not shown).

The cvt2, 3, 5, 6, and 7 Mutants Are Phenotypically Distinct from vps Mutants

As stated earlier, there are two different pathways that are capable of targeting resident proteins to the yeast vacuole (Fig. 1): the secretory pathway and cytoplasm to vacuole targeting. It is possible that the *cvt* mutants define a pathway that partially overlaps with the secretory pathway. Many of the *vps* mutants were identified on the basis of missorting of a vacuolar protein that transits through the secretory pathway. Therefore, one of the defining characteristics of the *vps* mutants is that a significant proportion of the accumulated precursor vacuolar proteins are secreted outside of the cells to the medium. To test the *cvt* mutants for CPY secretion, cells were labeled for 20 min then subjected to a 60-min chase. Each sample was then divided into cell and medium fractions and the extracts immunoprecipitated with antiserum to CPY as described in Materials and Methods. *vps15*, a class A mutant (Banta et

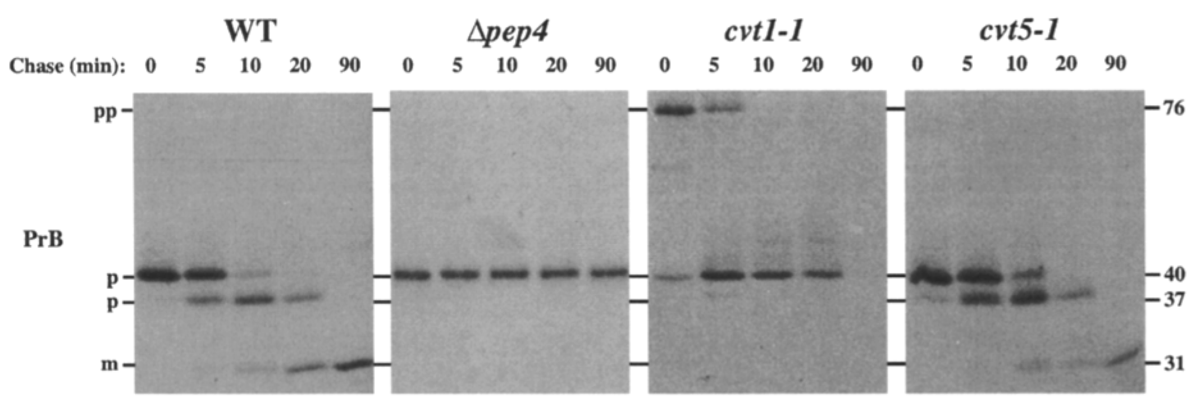

Figure 5. cvt1 is defective in synthesis and maintenance of the vacuolar endoprotease PrB. WT (SEY6211 transformed with pRN1), Δ*pep4* (SEY2101 Δ*pep4* transformed with pRN1), THY14 (*cvt1-1*), and THY313 (*cvt5-1*) cells were labeled for 5 min using [^{35}S]methionine, followed by a nonradioactive chase for the times indicated. Radiolabeled cell extracts were immunoprecipitated with antiserum to PrB as described in Materials and Methods. The designations pp, p, and m refer to the prepro, precursor, and mature forms of PrB. Numbers at the right indicate the molecular mass in kD.

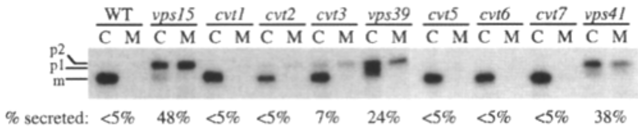

Figure 6. The *cvt* mutants do not secrete CPY. Wild-type (SEY6210), *vps* and *cvt* mutant cells were labeled for 20 min with [^{35}S]methionine in the presence of 2.5 mg/ml BSA. Chase was initiated by the addition of methionine/cysteine (1:2 mM) and 0.2% yeast extract and continued for 60 min. Samples were separated into cell (*C*) and medium (*M*) fractions by a 10-s centrifugation at 12,500 *g* and precipitated in 10% TCA. Radiolabeled extracts were immunoprecipitated with antiserum to CPY as described in Materials and Methods. The positions of the precursor and mature forms of CPY are as indicated.

al., 1988), was used as a control for *vps*-type secretion; nearly half of the CPY present is secreted from these cells (Fig. 6). This is in agreement with the high levels of secretion seen previously in this strain (Robinson et al., 1988). As expected, the alleles of *vps39* and *vps41* isolated by our screen also secrete a significant proportion of this protease. Strains bearing alleles of *cvt1, 2, 5, 6,* and *7* secreted less than 5% of the CPY to the medium fraction, which is comparable to the secretion levels observed in the wild-type parental strain. The mutant *cvt3* secretes a detectable level of CPY to the medium (7%), but this is significantly lower than that reported for mutants recognized as defective in secretory pathway-mediated vacuolar protein sorting (Raymond et al., 1992*a*).

Kinetics of Protein Maturation in cvt Strains

Steady-state analysis indicated that most of the *cvt* mutants were not defective for maturation of vacuolar proteins that transit through the secretory pathway (Fig. 3) and do not secrete significant amounts of CPY to the medium (Fig. 6) as would be expected in a *vps* mutant. To further examine the specificity of the *cvt* mutants, we carried out a kinetic analysis of vacuolar protein maturation (Fig. 7). Whole cells were labeled 5 min with [^{35}S]methionine and subjected to nonradioactive chase for the times indicated. Samples were then analyzed for protein modifications by immunoprecipitation as stated in Materials and Methods.

API has a long half-time for processing (30–45 min) in comparison to secretory pathway targeted proteins (Klionsky et al., 1992), and was almost completely matured at the 90-min chase point in wild-type cells (Fig. 7). In the Δ*pep4* control, mature API was not observed (Fig. 7). This was also true in all the *cvt* mutants, even when the chase was extended to six hours (Fig. 7 and data not shown). The *cvt1* strain accumulates variable amounts of intermediate sized proteins at steady-state that react with API antiserum (Fig. 3). The larger of these forms can be seen as a very faint band in the 90-min chase point of *cvt1-1* (Fig. 7).

PrA and CPY are known to transit through the secretory pathway en route to the vacuole, and display processing half-times of 6–10 min (reviewed in Klionsky et al., 1990; Raymond et al., 1992*b*). Processing of PrA was essentially indistinguishable from wild-type in all but one of the *cvt* strains; maturation of PrA in *cvt1* mutants was both slightly slower than that observed in wild-type, and the "mature" band migrated at 43 kD (m*) rather than 42 kD, as we had observed in the steady-state analysis (Fig. 3). This is in agreement with identification of the *cvt1* mutant as defective in PrB function (Hirsch et al., 1992).

In PrA-deficient mutants, CPY is targeted correctly but not matured beyond its Golgi-processed form (Fig. 7, 69 kD) (Ammerer et al., 1986). The *cvt* mutants showed a slight increase in the half-time of processing of CPY (Fig. 7). However, by the 20-min chase point, most of the *cvt* mutants (excluding *vps39* and *41*) accumulate 100% mature CPY (Fig. 6). We do not consider this to be a significant increase, as there is no effect on PrA processing. It should be noted that *cvt3* accumulates a very low amount of p2 CPY even at very long (90 min) chase points. This is

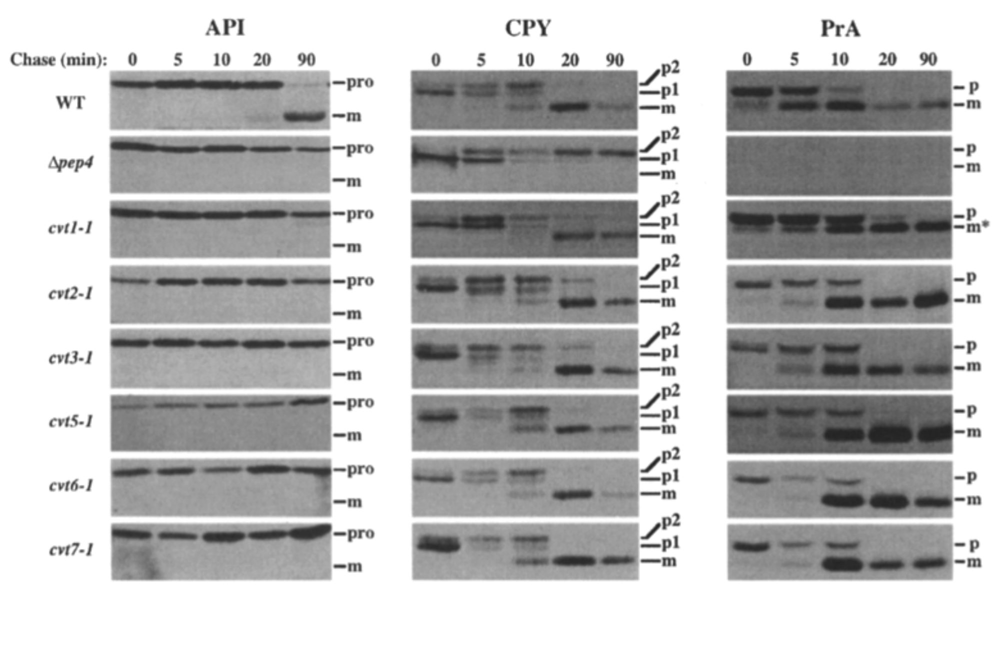

Figure 7. Most *cvt* mutants have specific defects in API maturation. WT (SEY6211 harboring pRN1), Δ*pep4* (SEY2101 Δ*pep4* harboring pRN1), THY14 (*cvt1-1*), THY193 (*cvt2-1*), THY119 (*cvt3-1*), THY313 (*cvt5-1*), THY144 (*cvt6-1*), and THY154 (*cvt7-1*) cells were labeled for 5 min using [^{35}S]methionine, followed by a nonradioactive chase as for the times indicated. Radiolabeled cell extracts were immunoprecipitated with antisera to mAPI, CPY, and PrA as described in Materials and Methods. Positions of each precursor and mature protein species are shown. m* indicates the position of 43-kD mature PrA in PrB-deficient cells.

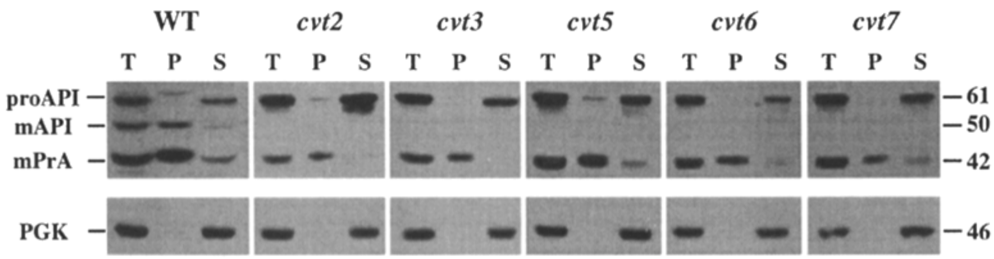

Figure 8. Mutants in *cvt2, 3, 5, 6,* and *7* accumulate proAPI outside of the vacuole. WT (SEY6210 with pRN1) and the specified *cvt* strains were grown to an OD_{600} of ~1, and 10–20 OD_{600} units harvested. The cells were converted to spheroplasts, subjected to osmotic lysis, and samples prepared as stated in Materials and Methods. Approximately 0.2 OD_{600} (anti-API and anti-PrA blot) or 0.02 OD_{600} equivalents (anti-PGK blot) of protein from each fraction were separated on two identical 10% SDS-PAGE gels, transferred to PVDF membrane, and probed as described in Materials and Methods. Positions of each protein species are shown. Numbers at the right indicate the molecular mass in kD. The highest molecular mass band (~63 kD) found in the total and pellet lanes of the WT sample is not API-specific. *T*, total before osmotic lysis; *P*, membrane pellet from 12,500 *g* centrifugation; *S*, supernatant from 12,500 *g* centrifugation.

in concurrence with its secretion of a low level of p2 CPY as seen in Fig. 6.

Subcellular Localization of Precursor API Indicates the cvt Mutants Block API Targeting to the Vacuole

Two types of mutants would show a significant accumulation of precursor API in our screen. The first are those that faithfully target API to the vacuole, but are defective in its maturation. The *cvtl* complementation group is an example of this type of mutation. The second class of mutations we expected to isolate is that which contains mutants blocked in API targeting to the vacuole or translocation across the vacuolar membrane. The fundamental phenotypic difference between these two types of mutants is the subcellular location of accumulated proAPI. In the first type we would expect the precursor to be inside of the vacuole, whereas mutants of the second type would accumulate proAPI outside the vacuole.

To distinguish between these two types of mutants, we devised a differential lysis fractionation procedure to examine the subcellular localization of API. Cells were spheroplasted, then subjected to gentle osmotic lysis to break the plasma membrane while leaving the vacuole membrane intact. A pellet fraction containing vacuoles, and a supernatant fraction, were prepared by differential centrifugation. The localization of API was determined by Western analysis. PrA was used as a marker for soluble vacuolar proteins, and phosphoglycerate kinase (PGK) as a marker for soluble cytosolic proteins. The osmotic lysis method employed typically released >95% of the detected PGK into the supernatant in all samples examined, while retaining >70% of the PrA in the pelleted fraction. In wild-type cells, greater than 90% of the accumulated mature API protein is found in the pellet (Fig. 8). Some precursor API can be observed in this preparation and is in the supernatant fraction. The amount of precursor observed in wild-type cells subjected to this lysis procedure varies from 30–50% of the total API detected in the sample. We believe that the accumulation of proAPI in this experiment is due to preparation of spheroplasts; if a sample of the cells is examined by Western blot analysis before spheroplasting, the level of proAPI in the wild-type strain is consistently <10% of the total API detected (see Fig. 2).

In cells with a disrupted copy of proteinase A (Δ*pep4*), API accumulates as the precursor-size band (61 kD) and is found in the pellet fraction; this is also true for alleles of *cvtl* (data not shown). In both of these strains, precursor API is targeted to the vacuole faithfully, but the vacuole lacks the functional protease necessary to convert API to the mature form. In fractionation experiments performed on the remaining *cvt* mutants, the accumulated proAPI did not colocalize with the vacuolar marker PrA, but rather was found in the 12,500 *g* supernatant fraction as was the cytosolic marker PGK (Fig. 8). When the supernatant fraction was subjected to a 100,000 *g* spin to sediment any small vesicles that might remain, proAPI was still recovered entirely in the supernatant fraction (data not shown). Together, these data demonstrate that API does not reach the vacuole in *cvt 2, 3, 5, 6,* and *7*, and also suggest that it is not trapped in a vesicular intermediate.

To further address the location of accumulated proAPI in *cvt* mutants, we determined its accessibility to protease. In this experiment, osmotically lysed spheroplasts were subjected either to a mock protease treatment, or to treatment with proteinase K in the presence and absence of the detergent Triton X-100. While mature API is resistant to digestion by proteinase K (see Fig. 9, *WT* lanes), API precursor can be degraded to lower molecular mass forms (see Fig. 9, Δ*pep4* + *Tx-100*). In both the Δ*pep4* strain and *cvtl*, the majority of accumulated pro-API is protease-protected unless membrane integrity is compromised by the addition of detergent (Fig 9); the lower molecular mass forms of API detected in the absence of detergent are due to a small amount of vacuole lysis. Protease-protected precursor API is the result that we expect if API is unprocessed inside the vacuole, as is the case here, or is localized inside a small vesicle. In *cvt2, 3, 5, 6,* and *7* as well as our *vps39* and *41* alleles, proAPI was accessible to protease even in the absence of detergent (Fig. 9), indicating it is not protected in a membrane-bound compartment. It is still possible that the precursor was located within a vesicular compartment before osmotic lysis, but we consider this unlikely because the vacuole itself is one of the most osmotically sensitive organelles, and its integrity is preserved in this assay (see Fig. 9, Δ*pep4* and *cvtl* lanes). Together, these data indicate that the five unique *cvt* mutants cause a specific block in targeting of API to and/or into the vacuole itself.

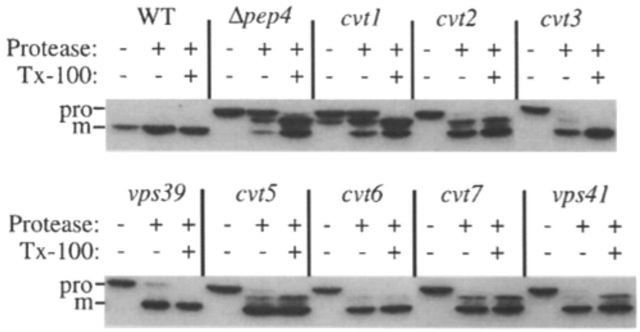

Figure 9. The nonvacuolar proAPI accumulated in *cvt* mutants is not enclosed in a membrane-bound compartment. The yeast strains specified were spheroplasted and subjected to differential osmotic lysis as in described in Materials and Methods. 2 OD_{600} equivalents of lysed spheroplasts were subjected either to a mock protease treatment or to treatment with 50 μg/ml proteinase K for 30 min on ice in the presence or absence of 0.2% Triton X-100. The positions of precursor and mature API are indicated.

Discussion

In this study, we have identified mutants in the cytoplasm to vacuole targeting pathway using API as a model protein. We produced antiserum that specifically recognizes the pro-region of API, and used this to screen for yeast mutants that accumulate high levels of the precursor form. Five identified complementation groups do not significantly affect vacuolar delivery of proteins that are known to transit through the secretory pathway. These mutants are specific for API targeting and are unique to this search.

Several types of defects could cause accumulation of proAPI in the cell, and would therefore be detected by our screen. For instance, as the pro-region of API is removed by the hydrolase PrB, we expected that defects in the *PRB1* gene would lead to accumulation of proAPI. Indeed, our screen identified six mutants that are alleles of *PRB1*. Alleles in this complementation group (*cvt1*) display aberrant accumulation of PrB itself, either accumulating no PrB at steady-state as seen in *cvt1-1*, or low levels of precursor bands in strains bearing other alleles (data not shown). The proAPI accumulation phenotype in this complementation group, as typified by *cvt1-1*, could be overcome by transforming the cells with a wild-type copy of the *PRB1* gene (data not shown). Because PrB maturation is dependent on proteinase A, we expected to isolate mutants in *pep4*. The absence of a *pep4* mutant, together with our allele distribution, suggests that we have not isolated mutants in all of the genes involved in API targeting.

We also expected our screen to identify mutants that severely disrupt the structure and maintenance of the vacuole itself, such as certain *vps* mutants. In fact, we found that two of the *cvt* mutants isolated in this study were allelic to previously identified *vps* class B mutants. Class B mutants accumulate fragmented membrane structures that still share many characteristics with wild-type vacuoles. It has previously been noted that class B *vps* mutants can be divided into two sub-groups based on subtle differences detectable by light and electron microscopy (Raymond et al., 1992*a*). Interestingly, the two alleles identified here, *vps39* and *vps41*, are both in the second sub-group of class B mutants. This sub-group is characterized by small vacuole fragments scattered throughout the cell, which show diffuse staining either with quinacrine or by immunofluorescence with antisera to the 60-kD subunit of the vacuolar ATPase (Raymond et al., 1992*a*). It is interesting to note that *vps* class B mutants in the first sub-group (*vps5* and *17*) do not have a major effect on API sorting (Klionsky et al., 1992). It is possible that the second sub-group of class B mutants is specifically involved in the process of API import, as the accumulated precursor API in these mutants is not contained within a membrane-bound compartment (Fig. 9). However, the defect in protease maturation is not limited to API; CPY and PrA are accumulated as precursor forms and/or secreted from the cells (Fig. 5). We therefore propose that these strains have pleiotropic defects in vacuolar protein delivery and are not specifically defective in cytoplasm to vacuole targeting.

Five of our complementation groups are specifically defective for the targeting and translocation of API. This can be seen in the protein maturation defect, examined both kinetically and at steady-state, as well as the cytoplasmic localization of the accumulated proAPI. The specific block of API maturation in *cvt2, 3, 5, 6,* and *7* suggests that these gene products have a direct role in targeting this protein to the vacuole. Work is currently underway to clone these genes by complementation of the proAPI accumulation phenotype. Identification of these gene products will help elucidate the specific mechanism that mediates the cytoplasm to vacuole targeting pathway.

The actual mechanism of protein translocation employed in the targeting of API is still open to question. It has been shown that API is posttranslationally localized to the vacuole (Klionsky et al., 1992), and therefore the import machinery must be competent to translocate the full length polypeptide. By analogy to more fully elucidated systems, this could occur via one of three general methods: (*a*) direct membrane translocation via a small active transporting protein such as an ATPase binding cassette (ABC) transporter (Higgins, 1992; Ortiz et al., 1995) or a large complex of proteins as seen in prokaryotic secretion (Wickner et al., 1991) and ER translocation (Rapoport, 1992); (*b*) by vesicle formation in the cytoplasm followed by fusion with the target organelle, as seen in autophagy; and (*c*) by membrane invagination leading to vesicle formation, as occurs during endocytosis and possibly peroxisomal import (McNew and Goodman, 1994). Each type of transport would require a certain set of proteins to mediate the translocation event.

Direct membrane translocation of API is appealing in that it agrees with the observation that precursor API is not detected inside a membrane-bound compartment before its entrance into the vacuole in the wild-type strain (Klionsky et al., 1992). Similarly the accumulated precursor is not protected from exogenously added protease in the *cvt* mutants (Fig. 9). A direct translocation mechanism would require one or more integral membrane protein(s) in the vacuolar membrane, as well as possible associated peripheral proteins. We might also expect the involvement of cytosolic and/or vacuolar chaperones, such as a member of the hsp70 class (Lindquist and Craig, 1988; Terlecky et al., 1992; Wickner, 1994). These factors have been shown to be necessary both for maintaining proteins in translocation-competent forms and for assisting in the import process (Chirico et al., 1988; Deshaies et al., 1988). This scenario is compatible with the observation that much of the newly synthesized API is not sorted correctly when overproduced (T. Harding and D. Klionsky, unpublished data). Overproduction may titrate out a critical cytosolic factor, thereby allowing proAPI to misfold into translocation-incompetent structures.

Autophagy, on the other hand, offers an intriguing alternative to direct membrane translocation of proAPI. Autophagy in yeast is known to transport proteins and organellar fragments destined for degradation from the cytosol into the vacuolar lumen. This process involves the formation of a cytosolic vesicle (autophagosome), and its subsequent fusion to the vacuole membrane (Takeshige et al., 1992; Thumm et al., 1994). This is a very slow process, with a half-time on the order of several hours (Noda et al., 1995). The transport time for API, however, is much more rapid, having a half-time of 30–45 min. In addition, autophagy in yeast is regulated through nutrient sensing (Takeshige et al., 1992), and increases during starvation. In contrast, import of API is maximal during optimal growth conditions (unpublished results). These considerations argue against a mechanism wherein API is a passive substrate for the autophagic machinery.

The third possible mechanism involves precursor binding to the vacuolar membrane, followed by invagination similar to that seen in receptor-mediated endocytosis. Though fluid phase endocytosis is a relatively slow process, molecules such as the yeast mating factors are endocytosed very rapidly and efficiently upon interaction with cell surface localized receptors (Jenness et al., 1983; Jenness and Spatrich, 1986; Sprague, 1991). If such a system occurs in API transport, the requirement for interaction with a limiting number of receptor molecules would explain the observed saturability of this pathway. Direct uptake at the vacuolar membrane would not necessarily require the involvement of a cytosolic chaperone, as API could fold to very nearly its final tertiary structure before its interaction with the receptor. It has recently been found that the yeast peroxisome can directly import an oligomeric protein (McNew and Goodman, 1994). One of the models that was proposed to explain this requires the interaction of the oligomeric protein with a receptor on the surface of the target organelle, followed by an as yet undetermined mode of movement of the importing protein into the peroxisome. It is possible that a similar mechanism occurs in API targeting. We are investigating the mechanism of API localization to determine which elements of these three general processes are utilized in the cytoplasm to vacuole targeting pathway.

We would like to thank Dr. E. Jones for the generous gift of yeast strains deficient in various proteases, the plasmid carrying the gene for PrB, and antiserum against PrB. We also thank Drs. S. Emr, R. Schekman, L. Weisman, A. Franzusoff, S. Ferro-Novick, and R. Deshaies for providing various other strains used in our complementation tests. We would like to thank Dr. R. Schekman for the suggestions that led to the design of the mutant screen used in this paper. Antisera to PGK was a gift from Dr. J. Thorner. Special thanks goes to K. C. McFarland, for advice and assistance in figure preparation, and to B. Garrison, for technical assistance during backcrosses and complementation tests. We also acknowledge A. Hefner-Gravink and J. Tomashek for reading the manuscript during preparation, and being unstinting in their constructive criticisms.

This material is based upon work supported by a National Science Foundation Graduate Research Fellowship to T. M. Harding, and Public Health Service grant DK43684 to D. J. Klionsky from the National Institutes of Health.

Received for publication 1 June 1995 and in revised form 7 August 1995.

References

Aitchison, J. D., W. M. Nuttley, R. K. Szilard, A. M. Brade, J. R. Glover, and R. A. Rachibinski. 1992. Peroxisome biogenesis in yeast. *Mol. Microbiol.* 6: 3455–3460.

Ammerer, G., C. P. Hunter, J. H. Rothman, G. C. Saari, L. A. Valls, and T. H. Stevens. 1986. *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. *Mol. Cell. Biol.* 6:2490–2499.

Baba, M., K. Takeshige, N. Baba, and Y. Ohsumi. 1994. Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization. *J. Cell Biol.* 124:903–913.

Bankaitis, V. A., L. M. Johnson, and S. D. Emr. 1986. Isolation of yeast mutants defective in protein targeting to the vacuole. *Proc. Natl. Acad. Sci. USA.* 83: 9075–9079.

Bankaitis, V. A., D. E. Malehorn, S. D. Emr, and R. Greene. 1989. The *Saccharomyces cerevisiae SEC14* gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi. *J. Cell Biol.* 108:1271–1281.

Banta, L. M., J. S. Robinson, D. J. Klionsky, and S. D. Emr. 1988. Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. *J. Cell Biol.* 107:1369–1383.

Baum, P., J. Thorner, and L. Honig. 1978. Identification of tubulin from the yeast *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA.* 75:4962–4966.

Bohni, P. C., G. Daum, and G. Schatz. 1983. Import of proteins into mitochondria. Partial purification of a matrix-located protease involved in cleavage of mitochondrial precursor polypeptides. *J. Biol. Chem.* 258:4937–4943.

Chang, Y.-H., and J. A. Smith. 1989. Molecular cloning and sequencing of genomic DNA encoding aminopeptidase I from *Saccharomyces cerevisiae. J. Biol. Chem.* 264:6979–6983.

Chirico, W. J., M. G. Waters, and G. Blobel. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature (Lond.).* 332: 805–810.

Chvatchko, Y., I. Howald, and H. Riezman. 1986. Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell.* 46:355–364.

Cueva, R., N. Garcia-Alvarez, and P. Suarez-Rendueles. 1989. Yeast vacuolar aminopeptidase *yscI*: isolation and regulation of the *APE1 (LAP4)* structural gene. *FEBS Lett.* 259:125–129.

Davis, N. G., J. L. Norecka, and G. F. J. Sprague. 1993. *cis*- and *trans*-acting functions required for endocytosis of the yeast pheromone receptors. *J. Cell Biol.* 122:53–65.

Deshaies, R. J., and R. Schekman. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* 105:633–645.

Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.).* 332:800–805.

Emr, S. D., A. Vassorotti, J. Garrett, B. L. Geller, M. Takeda, and M. G. Douglas. 1986. The amino terminus of the yeast F_1-ATPase B-subunit precursor functions as a mitochondrial import signal. *J. Cell Biol.* 102:523–533.

Franzusoff, A., and R. Schekman. 1989. Functional compartments of the yeast Golgi apparatus are defined by the *sec7* mutation. *EMBO J.* 8:2695–2702.

Gallagher, S. 1994. Visualization with luminescent substrates. *In* Current Protocols in Molecular Biology. Vol. 2. F. Ausubel, R. Brent, R. Kingsten, D. Moore, J. G. Seidna, J. Smith, and K. Struhl, editors. John Wiley and Sons, Inc., NY. 10.8.11–13.

Glick, B., and G. Schatz. 1991. Import of proteins into mitochondria. *Annu. Rev. Genet.* 25:21–44.

Guthrie, C., and G. R. Fink. 1991. Guide to yeast genetics and molecular biology. *In* Methods in Enzymology. Vol. 194. J. N. Abelson and M. I. Simon, editors. Academic Press, Inc., NY, 1–905.

Hannavy, K., S. Rospert, and T. Schatz. 1993. Protein import into mitochondria: a paradigm for the translocation of polypeptides across membranes. *Curr. Opin. Cell Biol.* 5:694–700.

Hendrick, J. P., and F.-U. Hartl. 1993. Molecular chaperone functions of heat-shock proteins. *Annu. Rev. Biochem.* 62:349–384.

Higgins, C. F. 1992. ABC Transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* 8:67–113.

Hirsch, H. H., H. H. Schiffer, and D. H. Wolf. 1992. Biogenesis of the yeast vacuole (lysosome): proteinase *yscB* contributes molecularly and kinetically to vacuole hydrolase-precursor maturation. *Eur. J. Biochem.* 13:1–10.

Jenness, D. D., and P. Spatrich. 1986. Down regulation of the alpha-factor pheromone receptor in *S. cerevisiae. Cell.* 46:345–353.

Jenness, D. D., A. C. Burkholder, and L. H. Hartwell. 1983. Binding of a-factor pheromone to yeast a cells: Chemical and genetic evidence for an a-factor receptor. *Cell.* 35:521–529.

Klionsky, D. J., L. M. Banta, and S. D. Emr. 1988. Intracellular sorting and processing of a yeast vacuolar hydrolase: proteinase A propeptide contains vacuolar targeting information. *Mol. Cell. Biol.* 8:2105–2116.

Klionsky, D. J., P. K. Herman, and S. D. Emr. 1990. The fungal vacuole: Composition, function and biogenesis. *Microbiol. Rev.* 54:266–292.

Klionsky, D. J., R. Cueva, and D. S. Yaver. 1992. Aminopeptidase I of *Saccharomyces cerevisiae* is localized to the vacuole independent of the secretory pathway. *J Cell Biol.* 119:287–299.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature (Lond.).* 227:680–685.

Lindquist, S., and E. A. Craig. 1988. The heat-shock proteins. *Annu. Rev. Genet.* 22:631–677.

Lyons, S., and N. Nelson. 1984. An immunological method for detecting gene expression in yeast colonies. *Proc. Nat. Acad. Sci. USA.* 81:7426–7430.

McNew, J. A., and J. M. Goodman. 1994. An oligomeric protein is imported into peroxisomes *in vivo. J. Cell Biol.* 127:1245–1257.

Mechler, B., H. H. Hirsch, H. Muller, and D. H. Wolf. 1988. Biogenesis of the yeast lysosome (vacuole): biosynthesis and maturation of proteinase *ysc*B. *EMBO J.* 7:1705-1710.

Michaelis, S. 1993. STE6, the yeast a-factor transporter. *Semin. Cell Biol.* 4:17–27.

Moehle, C. M., M. W. Aynardi, M. R. Kolodny, F. J. Park, and E. W. Jones. 1987. Protease B of *Saccharomyces cerevisiae*: isolation and regulation of the *PRB1* structural gene. *Genetics.* 115:255–263.

Moehle, C. M., C. K. Dixon, and E. W. Jones. 1989. Processing pathway for protease B of *Saccharomyces cerevisiae. J. Cell Biol.* 108:309–324.

Morano, K. A., and D. J. Klionsky. 1994. Differential effects of compartment deacidification on the targeting of membrane and soluble proteins to the vacuole in yeast. *J. Cell Sci.* 107:2813–2824.

Newman, A. P., J. Shim, and S. Ferro-Novick. 1990. *BET1, BOS1* and *SEC22* are members of a group of interacting yeast genes required for transport from the endoplasmic reticulum to the Golgi complex. *Mol. Cell. Biol.* 10: 3405–3414.

Noda, T., A. Matsuura, Y. Wada, and Y. Osumi. 1995. Novel system for monitoring autophagy in the yeast *Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun.* 210:126–132.

Ortiz, D. F., T. Ruscitti, K. F. McCue, and D. W. Ow. 1995. Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. *J. Biol. Chem.* 270:4721–4728.

Pryer, N. K., L. J. Wuestehube, and R. Schekman. 1992. Vesicle mediated protein sorting. *Annu. Rev. Biochem.* 61:471-516.

Rapoport, T. A. 1992. Transport of proteins across the endoplasmic reticulum membrane. *Science (Wash. DC).* 258:931–936.

Raths, S., J. Rohrer, F. Crausaz, and H. Riezman. 1993. *end3* and *end4*: Two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae. J. Cell Biol.* 120:55–65.

Raymond, C. K., I. Howald-Stevenson, C. A. Vater, and T. H. Stevens. 1992*a*. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E *vps* mutants. *Mol. Biol. Cell.* 3:1389–1402.

Raymond, C. K., C. J. Roberts, K. E. Moore, I. Howald, and T. H. Stevens. 1992*b*. Biogenesis of the vacuole in *Saccharomyces cerevisiae. Int. Rev. Cyt.* 139:59–120.

Riezman, H. 1985. Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. *Cell.* 40:1001–1009.

Roberts, C. J., C. K. Raymond, C. T. Yamashiro, and T. Stevens. 1991. Methods for studying the yeast vacuole. *In* Guide to Yeast Genetics and Molecular Biology. Vol. 194. C. Guthrie and G. R. Fink, editors. Academic Press, Inc., San Diego, CA. 644–661.

Robinson, J. S., D. J. Klionsky, L. M. Banta, and S. D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8:4936–4948.

Rothblatt, J. A., R. J. Deshaies, S. L. Sanders, G. Daum, and R. Schekman. 1989. Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* 109:2641–2652.

Rothman, J. H., I. Howald, and T. H. Stevens. 1989. Characterization of genes required for protein sorting and vacuolar function in the yeast *Saccharomyces cerevisiae. EMBO J.* 8:2057–2065.

Rothman, J. H., and T. H. Stevens. 1986. Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. *Cell.* 47:1041–1051.

Singer, B., and H. Riezman. 1990. Detection of an intermediate compartment involved in transport of alpha-factor from the plasma membrane to the vacuole in yeast. *J. Cell Biol.* 110:1911–1922.

Singer-Kruger, B., R. Frank, F. Crausaz, and H. Reizman. 1993. Partial purification and characterization of early and late endosomes from yeast. *J. Biol. Chem.* 268:14376–14386.

Sprague, G. F. J. 1991. Signal transduction in yeast mating: receptors, transcription factors, and the kinase connection. *Trends Genet.* 7:393–398.

Stack, J. H., and S. D. Emr. 1993. Genetic and biochemical studies of protein sorting to the yeast vacuole. *Curr. Opin. Cell Biol.* 5:641–646.

Stevens, T., B. Esmon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* 30:439–448.

Takeshige, K., M. Baba, S. Tsuboi, T. Noda, and Y. Ohsumi. 1992. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J. Cell Biol.* 119:301–311.

Terlecky, S. R., H.-L. Chiang, T. S. Olson, and J. F. Dice. 1992. Protein and peptide binding and stimulation of *in vitro* lysosomal proteolysis by the 73-kDa heat shock cognate protein. *J. Biol. Chem.* 207:9202–9209.

Thumm, M., R. Egner, M. Koch, M. Schlumpberger, M. Straub, M. Veenhuis, and D. H. Wolf. 1994. Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae. FEBS Lett.* 349:275–280.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Vida, T. A., and S. D. Emr. 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* 128:779–792.

Vida, T. A., G. Huyer, and S. D. Emr. 1993. Yeast vacuolar proenzymes are sorted in the late Golgi complex and transported to the vacuole via a prevacuolar endosome-like compartment. *J. Cell Biol.* 121:1245–1256.

Weisman, L. S., S. D. Emr, and W. Wickner. 1990. Mutants of *Saccharomyces cerevisiae* that block intervacuole vesicular traffic and vacuole division and segregation. *Proc. Nat. Acad. Sci. USA.* 87:1076–1080.

Wickner, W. T. 1994. How ATP drives proteins across membranes. *Science (Wash. DC).* 266:1197–1198.

Wickner, W., A. J. M. Driessen, and F.-U. Hartl. 1991. The enzymology of protein translocation across the *Escherichia coli* plasma membrane. *Annu. Rev. Biochem.* 60:101–124.

Yoshihisa, T., and Y. Anraku. 1990. A novel pathway of import of a-mannosidase, a marker enzyme of vacuolar membrane, in *Saccharomyces cerevisiae. J. Biol. Chem.* 265:22418–22425.