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). 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 organelar 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, nutrient-sensitive proteins are internalized in response to a drop in nutrient concentration. (c) Membrane proteins that exit the secretory pathway are destined for degradation. These proteins are synthesized in an N-terminal signal peptide that is removed by signal peptidase following translocation across the ER (Inouye et al., 1992). Because peptides are not removed at the plasma membrane, these proteins are delivered to the vacuole. (d) The vacuolar targeting sequence found in some secretory pathway independent proteins determines the delivery of these proteins to the vacuole. The vacuolar targeting sequence of these proteins is not dependent on the secretory pathway (Klionsky et al., 1992). A defined role for the vacuole in the transport of secretory pathway-independent proteins has not been established.

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.
tionally, it may also need to interact with cytosolic chaperones 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 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 autophagy (Takeshige et al., 1992; Baba et al., 1994; Thumm 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.

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.

Materials and Methods

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). Expre35S$^{35}$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 (Swedesboro, NJ), acrylamide and proteinase K were from Boehringer Mannheim Biochemical Corp. (Indianapolis, IN), 0.45 μS 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 from Millipore (Marlborough, MA). Antisera to PRB and PrP 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 resulting blunt-ended fragment was ligated into the Smal 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td>SEY6210</td>
<td>MATa leu2-3, 112 ura3-52 his3-D200 trpl-D901 lys2-801 suc2-D9</td>
<td>Robinson et al., 1988</td>
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<tr>
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<td>Robinson et al., 1988</td>
</tr>
<tr>
<td>vps16-8, 8-35</td>
<td>derived from SEY6210 of SEY6211</td>
<td>Robinson et al., 1988</td>
</tr>
<tr>
<td>SEY2101</td>
<td>MATa leu2-3, 112 ura3-52 ade2-101 suc2-D9 pep4::LEU2</td>
<td>Bankaitis et al., 1992</td>
</tr>
<tr>
<td>DYY101</td>
<td>MATa leu2-3, 112 ura3-52 ade2-101 suc2-D9</td>
<td>Bankaitis et al., 1992</td>
</tr>
<tr>
<td>SF838-10</td>
<td>MATa ade6 his4-519 ura3-52 leu2,3-112 pep4-3</td>
<td>Rothman and Stevens, 1986</td>
</tr>
<tr>
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<td>Rothman and Stevens, 1986</td>
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<td>vps7, 36-39,41, 43-46</td>
<td>derived from SF838-9DR2L1, SF838-90, or SF838-10</td>
<td>Rothman and Stevens, 1986</td>
</tr>
<tr>
<td>SF838-9DR2L1</td>
<td>MATa his4-519 ura3-52 leu2,3-112 pep4-3</td>
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<td>SF838-10</td>
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<td>Rothman and Stevens, 1986</td>
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**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 ~10·10^6 cells/ml. Ethylmethane sulfonate (EMS) was added to a final concentration of 30 μg/ml, and the cells were incubated on a roller drum for 1 hour at 30°C. The cells were washed twice 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 37°C for 3 h. Filters were then placed on Whatman paper saturated with 67 mM potassium phosphate, 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 mixd on a vortex mixer for 1 min. Each sample was then heated 10 min at 65°C and 500 μl of fresh lys 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 then 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, and 1:30,000, and antiserum to PGK at 1:75,000 in "I-TBS with 0.5% nonfat milk. Acridine coral 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 of fresh lys 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 then 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 1:50,000, 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 horsedarish 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 mg 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 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 OD600 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 (100 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. The samples were then incubated at 30°C with continuous shaking (200–250 rpm), then harvested by centrifugation and resuspended in 1 M sorbitol, 20 mM Pipes, pH 6.8 (spheroplasting buffer), to an OD600 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 OD600 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 OD600 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 (Barkaitis et al., 1986;
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 I 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 antiserum, 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-

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**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 cvt6 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 effects.

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 hydrolyases 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 hydrolyases 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 in vacuole 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 depen-

dent 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., 1992b; Stuck 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., 1992a). Recent studies have also 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., 1992a), 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 [35S]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). Vacular 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.](image-url)
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.

**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 (7%), but this is significantly lower than that reported for mutants recognized as defective in secretory pathway-mediated vacuolar protein sorting (Raymond et al., 1992a).

**Figure 6.** The cvt mutants do not secrete CPY. Wild-type (SEY6210), vps and cvt mutant cells were labeled for 20 min with [35S]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.

AP1 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 Δapep4 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., 1992b). 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), Δapep4 (SEY2101 Δapep4 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 [35S]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.
pared as stated in Materials and Methods. Approximately 0.2 OD₆₀₀ (anti-API and anti-PrA blot) or 0.02 OD₆₀₀ 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 APl-specific. T, total before blot; 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 cvt1 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 (Δpep4), API accumulates as the precursor-size band (61 kD) and is found in the pellet fraction; this is also true for alleles of cvt1 (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 cvt2, 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, Δpep4 + Tx-100). In both the Δpep4 strain and cvt1, 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 4f1 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, Δpep4 and cvt1 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 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 OD₆₀₀ of ~1, and 10–20 OD₆₀₀ units harvested. The cells were converted to spheroplasts, subjected to osmotic lysis, and samples prepared as stated in Materials and Methods. Approximately 0.2 OD₆₀₀ (anti-API and anti-PrA blot) or 0.02 OD₆₀₀ 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 APl-specific. T, total before blot; P, membrane pellet from 12,500 g centrifugation; S, supernatant from 12,500 g centrifugation.
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-f, 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-f, 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., 1992a). 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., 1992a). 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 gene products 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 organelar 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 vacuolar 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 vacular 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 Spathrich, 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 saturaury 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 such a 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.

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