Abstract. The Saccharomyces cerevisiae APEI gene product, aminopeptidase I (API), is a soluble hydrolase that has been shown to be localized to the vacuole. API lacks a standard signal sequence and contains an unusual amino-terminal propeptide. We have examined the biosynthesis of API in order to elucidate the mechanism of its delivery to the vacuole. API is synthesized as an inactive precursor that is matured in a PEP4-dependent manner. The half-time for processing is approximately 45 min. The API precursor remains in the cytoplasm after synthesis and does not enter the secretory pathway. The precursor does not receive glycosyl modifications, and removal of its propeptide occurs in a sec-independent manner. Neither the precursor nor mature form of API are secreted into the extracellular fraction in etr mutants or upon overproduction, two additional characteristics of soluble vacuolar proteins that transit through the secretory pathway. Overproduction of API results in both an increase in the half-time of processing and the stable accumulation of precursor protein. These results suggest that API enters the vacuole by a posttranslational process not used by most previously studied resident vacuolar proteins and will be a useful model protein to analyze this alternative mechanism of vacuolar localization.

In the yeast Saccharomyces cerevisiae, the vacuole is integrally involved in a wide array of physiological processes (reviewed in Klionsky et al., 1990). These include pH and osmoregulation, protein degradation and storage of amino acids, small ions, and polyphosphate. These diverse functions necessitate the presence in the vacuole of a specific group of proteins. As with all organelles, accurate and efficient delivery of the resident proteins is critical to that organelle's ability to carry out its designated role(s) in cellular metabolism. In a general sense, proteins arrive at most organelles through one of two primary routes: the proteins either remain in the cytoplasm after being synthesized and are translocated directly into the organelle, or they enter the secretory pathway and are delivered to subsequent organelles via vesicular intermediates. The vacuole is perhaps unique in that protein targeting to this organelle employs both of these processes. All but one of the characterized vacuolar proteins transit through a portion of the secretory pathway before being directed to the vacuole (Klionsky et al., 1990). This mechanism of vacuolar delivery has been carefully analyzed although many questions remain to be resolved. In general, proteins are cotranslationally translocated into the ER due to the presence of an amino-terminal signal sequence. These proteins transit to the Golgi complex, undergoing various glycosyl and/or proteolytic modifications. At the trans Golgi network, they are separated from other proteins utilizing the secretory pathway and are directed to the vacuole.

In contrast, the vacuolar hydrolase α-mannosidase appears to enter the vacuole directly from the cytoplasm (Yoshihisa and Anraku, 1990). Some proteins destined for degradation, such as fructose 1,6-bisphosphatase, may also enter the vacuole in this manner (Funaguma et al., 1985; Chiang and Schekman, 1991), although the specific mechanism in either case has not been elucidated. In addition, proteins may be delivered to the vacuole from the cell surface via endocytosis. The best studied example of this is the PEP4-dependent turnover of α-factor (Chvatchko et al., 1986; Jenness and Spatrich, 1986; Payne et al., 1988; Singer and Riezman, 1990). It is not known if other proteins are delivered to the vacuole in this way. At present, many aspects of these different targeting mechanisms have not been well characterized.

Many analyses of vacuolar protein biosynthesis and sorting were initiated through the identification of protease activities corresponding to this organelle. The use of a wide variety of substrates has allowed the characterization of over eight different vacuolar hydrolases (Jones, 1984; Suarez Rendueles and Wolf, 1988; Klionsky et al., 1990). There are four identified aminopeptidases in Saccharomyces cerevisiae that hydrolyze leucine substrates (Trumbly and Bradley, 1983). Of these, only aminopeptidase I (API; previously designated LAPIV) appears to be localized to the vacuole based on subcellular fractionation (Matile et al., 1971; Frey and Röhm, 1978). Initial studies of API characterized it as being a glycoprotein containing 12% carbohydrate (Metz...
It was also shown that API was synthesized as an inactive zymogen that was processed in a PEP4-dependent manner (Trumbly and Bradley, 1983; Cueva et al., 1989; Chang and Smith, 1989) like all other soluble vacuolar proteins that transit through the secretory pathway (Klionsky et al., 1990). The gene encoding API, APEI (previously designated LAP4), has been cloned and the nucleotide sequence determined (Cueva et al., 1989; Chang and Smith, 1989). The APEI gene codes for a protein of 514 amino acids containing four potential sites for N-linked glycosylation. Amino acid sequence analysis of the mature protein indicated the presence of a 45-residue propeptide. The propeptide of API, however, does not contain a consensus signal sequence cleavage site. Furthermore, it lacks any homology to a standard signal sequence because it is not hydrophobic. Instead, a region of the amino terminus has the characteristics of an amphiphilic α-helix (Cueva et al., 1989; Chang and Smith, 1989).

API was presumed to transit through the secretory pathway because it appeared to be glycosylated. The initial studies regarding glycosylation, however, were not conclusive. The active enzyme is a multimeric protein having a molecular mass of ~600,000 D. The mature API monomer has a molecular mass of 50,000–51,000 D based in part on migration in SDS-PAGE (Metz and Röhm, 1976; Chang and Smith, 1989). The molecular mass based on the amino acid composition, however, predicts a protein of ~45,000 D (Metz and Röhm, 1976). This fact coupled with carbohydrate analyses led Metz and Röhm to assume that the additional 5 kD were derived from oligosaccharides. The presence of carbohydrate was not confirmed, however, by treatment with tunicamycin or binding to carbohydrate-specific lectins. To resolve these questions and to further characterize the nature of this protein, we have carried out a detailed immunological analysis of the biosynthesis and sorting of API. We have found that API is not glycosylated and does not enter the secretory pathway. The API precursor remains in the cytoplasm and appears to translocate directly into the vacuole with a half-time of ~45 min. These results indicate that API may enter the vacuole by a mechanism that is unique from that used by almost all of the characterized vacuolar proteins.

### Materials and Methods

#### Strains and Media

The Saccharomyces cerevisiae yeast strains used in this study are listed in Table I. Strains NAYII-17 and NAYIII-1 were generated from a cross between strain 1189 (Trumbly and Bradley, 1983) and strain DBY746 (Garcia-Alvarez et al., 1991). The vps mutants used are derivatives of strains SEY6210 (vps5-7, 17-9) or SEY6211 (vps1-3, 8–30, 15–14, 26–8) (Robinson et al., 1988). Strain BJ3044 was generously supplied by Elizabeth Jones (Carnegie Mellon University, Pittsburgh, PA). Standard methods (Sherman et al., 1979) were used to construct yeast strain DYY101 (see below). YPD medium (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose), and yeast nitrogen base (YNB) medium with the appropriate supplements were used for growing yeast.

#### Reagents

Lyticase was obtained from Eazogenetics (Corvalis, OR), Tran35S-label was from ICN Radiochemicals (Irvine, CA), DNA restriction and modifying enzymes were from New England Biolabs, Inc. (Beverly, MA), α2-macroglobulin was from Boehringer Mannheim Biochemicals (Indianapolis, IN), L-leucine β-naphthylamide was from Bachem (Philadelphia, PA), and Autofluor was from National Diagnostics, Inc. (Manville, NJ). SP6 polymerase, RNasin ribonuclease inhibitor and rNTPs were from Promega Biochemicals (Madison, WI). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Wheat germ lyase was generously supplied by William Ettinger and Steven Theg (Department of Botany, University of California, Davis). Antisera to PrA, CPY, and ALP were prepared as described previously (Klionsky et al., 1988; Klionsky and Emr, 1989). To produce antisemurum to API, we had two synthetic peptides made (Multiple Peptide Systems, San Diego, CA) based on the deduced amino acid sequence of the APEI gene. Peptides corresponding to amino acid residues 168–182 and 191–212 of API were separately conjugated at their COOH termini to keyhole limpet hemocyanin. Standard procedures were used to generate antisemurum in New Zealand White rabbits.

#### Table I. Yeast Strains

| Yeast strains | Genotype | Source |
|---------------|----------|--------|
| BJ3044        | MATa can1 lys2-801 prb1-Δ1.6 ura3-52 | Moehle et al., 1989 |
| DBY746        | MATα leu2-3,112 his31 ura3-52 tsp1-289 | Yeast Genetic Stock Center |
| DYY101        | MATα leu2-3,112 ura3-52 his3-Δ200 tsp1-Δ901 ade2-101 suc2-Δ9 GAL ape1::LEU2 | This study |
| NAYII-17      | MATα lap1 lap2 lap3 lap4 leu2-3,112 his3-Δ1 ura3-52 tsp1-289 | N. Garcia-Alvarez (University of Oviedo) |
| NAYIII-1      | MATα lap1 lap2 lap3 leu2-3,112 his3-Δ1 ura3-52 | N. Garcia-Alvarez |
| SEY2101       | MATα leu2-3,112 ura3-52 ade2-1 suc2-Δ9 | Err et al., 1983 |
| SEY2101Δpep4  | MATα leu2-3,112 ura3-52 ade2-1 suc2-Δ9 pep4::LEU2 | Klionsky et al., 1988 |
| SEY5187       | MATα sec18-1 leu2-3,112 ura3-52 suc2-Δ9 | Klionsky et al., 1988 |
| SEY6210       | MATα leu2-3,112 ura3-52 his3-Δ200 tsp1-Δ901 lys2-801 suc2-Δ9 GAL | Robinson et al., 1988 |
| SF274-3A      | MATα gal2 sec12-4 | C. Field and R. Schekman (University of California, Berkeley) |
| SF309-2C      | MATα gal2 sec23-1 | C. Field and R. Schekman |
Plasmid Construction

The APEI gene was cloned by complementation of the lap4 mutation as described previously (Cueva et al., 1989). One of the multicopy number (Cu circle) complementing plasmids, pRCA, contains a 4.7-kb BamHI fragment encoding APEI, cloned into the plasmid YEp3 (Nasmyth and Tatthell, 1980). The centromeric (CEN4 ARS1) plasmid pRL1 was constructed by cloning a 4.5-kb Sphl fragment encoding the entire APEI gene from pRCA into the Sphl site of YCp50 (Johnston and Davis, 1984). To carry out in vitro transcription/translation of APEI, a 1.69-kb DNA fragment containing the APEI gene was amplified using the polymerase chain reaction. Oligonucleotides containing a SalI site before nucleotide 1685 of the APEI gene (Cueva et al., 1989) were used for the reaction. The resulting fragment was ligated into pKK4 that had been restricted with SalI and HindIII. The plasmid pKK4 was supplied by Thomas Moore and Alan Bennett (Department of Vegetable Crops, University of California, Davis). This plasmid is a derivative of pBR322 (Melton et al., 1984) in which the Sphl site has been destroyed and the polylinker has been replaced with the polylinker from pUC19 (Yanisch-Perron et al., 1985).

Plasmid pAM1 (Kuranda and Robbins, 1987) encodes α-mannosidase on a multiple copy vector and was supplied by Dr. Michael Kuranda and Dr. Phillips Robbins (Massachusetts Institute of Technology). The multiple copy plasmids pDSY1 (Stevens et al., 1986) and pPA3 (Rothman et al., 1986), encoding CPY and Pra, respectively, were supplied by Dr. Tom Stevens (University of Oregon).

To disrupt the chromosomal APEI locus, the APEI gene was cloned into a derivative of pUC19 in which the EcoRI site had been destroyed. This was performed in two steps as follows: First, a 1.3-kb BamHI/Sall fragment from the COOH-terminal portion of the APEI gene was cloned into the pUC19 derivative. The resulting plasmid, pDSY1, was restricted with BamHI. Second, a 2.0-kb BamHI fragment from pRCA was ligated into the restricted BamHI site of pDSY1 to generate pDSY2 (Fig. 1). This plasmid, containing the entire APEI gene, was restricted with EcoRI to remove almost the entire APEI coding region, and the overlapping ends were blunt-ended with the Klenow fragment of DNA polymerase I. A XhoI/Sall fragment containing the LEU2 gene from the plasmid YEp3 (Nasmyth and Tatthell, 1980) was ligated into the blunt-ended EcoRI site of pDSY2 to generate the APEI disruption plasmid pDSY3 (Fig. 1). This plasmid was linearized with BamHI and Sall and used to transform yeast strain Joyce1210 to generate the chromosomal APEI deletion strain DYY101.

Cell Labeling and Immunoprecipitation

The procedures used for the preparation, labeling, fractionation, and immunoprecipitation of yeast cells and spheroplasts were modifications of procedures described previously (Klionsky et al., 1988; Robinson et al., 1988; Klionsky et al., 1992). Because of the long half-time of processing of API, it was necessary to carry out pulse-chase analyses using conditions where the cultures were at a relatively low initial density. This ensured that the cells were able to grow throughout the labeling and chase periods. Generally, cells were labeled at concentrations of 4–5 OD600 per ml and then diluted to 0.5 OD600 per ml by the addition of fresh medium for the nonradioactive chase. Chase solutions contained methionine (8 mM) and cysteine (4 mM).

To test for the presence of carbohydrate, cells were grown to mid-log phase and separated into two cultures. One culture was treated with tunicamycin (20 μg/ml final concentration) for 15 min prior to the addition of label. Cells were labeled for 30 min and then subjected to a nonradioactive chase for 0 or 120 min. At the end of the chase, the cells were precipitated with TCA, washed with acetone, dried, and resuspended in 100 μl MES/urea resuspension buffer (MURB; 50 mM sodium phosphate, 25 mM MES, pH 7.0, 1% SDS, 3 M urea, 0.5% β-mercaptoethanol, 1 mM sodium azide). Each sample was then divided in half and diluted with 800 μl of Tween 20 IP buffer (0.5% Tween 20, 50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 0.1 mM EDTA). One half was immunoprecipitated with antisera to API and CPY (total sample). The remaining half was diluted with 1 ml of a 1:20 reaction buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 1% Triton X-100 [TX-100]) and precipitated with Con A–sepharose essentially as described (Deshaies and Schekman, 1987; Baker et al., 1988). Con A precipitations were carried out using 30 μl of packed Con A–sepharose beads per sample. The supernatant fraction from the Con A precipitations was boiled for 4 min, centrifuged to remove insoluble material, and subjected to immunoprecipitation (supernatant sample). The pellets were washed twice with Con A reaction buffer and boiled in 100 μl MURB. The pellet samples were then diluted with 1 ml tween a reaction buffer and centrifuged, and the resulting supernatant fraction was immunoprecipitated with antisera to API and CPY (pellet sample).

Accessibility to exogenous protease was used to examine the location of precursor and mature API. Because mature API is resistant to proteolysis, the location of "mature" API was assessed by using spheroplasts prepared from a culture of strain Sey2101 (Δaep4). Spheroplasts were labeled for 20 min and subjected to a 2-min (precursor API) or 15-min ("mature" API) chase. After each chase point, the spheroplasts were centrifuged for 2 min at 3,000 rpm in the presence of 10 mM sodium azide. The spheroplast pellet was resuspended in 0.2 M imidazole, pH 6.5, 0.2 M sorbitol, 15% ficol and gently lysed by the addition of DEAE-dextran (Darr et al., 1975; Klionsky and Emr, 1989). Unlysed spheroplasts were removed by centrifugation. The supernatant fractions were divided into three aliquots, two of which received either pronase (200 μg/ml) or pronase and TX-100 (0.2%). The samples were incubated at 3°C for 30 min following precipitation with TCA. The samples were then immunoprecipitated with antisera to API and CPY.

To examine whether API is membrane-associated, a culture of strain Sey5187 was converted to spheroplasts and shifted to 37°C for 15 min. The spheroplasts were labeled at 32°C for 30 min followed by a nonradioactive chase for 30 min. The spheroplasts were pelleted by centrifugation and lysed by resuspension in 1.5 ml of lysis buffer (50 mM potassium phosphate, pH 7.5, 10 mM sodium azide, 100 μg/ml α-macroglobulin). Samples were divided into three aliquots, two of which received either saponin (0.4% final concentration) or TX-100 (0.1% final concentration). The samples were incubated at 4°C for 30 min, and then separated into supernatant and pellet fractions by centrifugation for 5 min at 12,300 g. The supernatant and pellet fractions were TCA precipitated and immunoprecipitated with antisera to CPY, API, and ALP.

In Vitro Synthesis of API

In vitro transcription of the APEI gene was carried out using the pKk4/APEI construct described above. The transcription reaction was carried out essentially as described (Melton et al., 1984) using 5 μg of DNA linearized with HindIII. In vitro translation of the mRNA from the transcription reaction was performed using wheat germ lysate as previously described (Theg et al., 1989). Tran35S-label was substituted for 3H-leucine.

Nondenaturing Polyacrylamide Gel Electrophoresis and Aminopeptidase Activity Staining

Soluble yeast extracts were prepared as described previously (Garcia-Alvarez et al., 1991). Extracts were electrophoresed under nondenaturing conditions as described by Davis (1964) using a 4% stacking gel and a 7.5% separating gel. The gels were stained for aminopeptidase activity as described (Hirsch et al., 1988), with L-leucine β-naphthylamide as the substrate.
Figure 2. Leucine aminopeptidase activity in wild-type and mutant strains. Soluble protein extracts were prepared from strains containing the indicated aminopeptidases. The extracts were electrophoresed under nondenaturing conditions and stained for activity using L-leucine/β-naphthylamide as the substrate as described in Materials and Methods. The strains and their relevant genotypes are as follows: (lane 1) SEY6210, API+; (lane 2) DY101, Δape1; (lane 3) DY101/pRNI, Δape1/API+; (lane 4) NAYIII-1, lap1 lap2 lap3 (API+); (lane 5) NAYII-17, lap1 lap2 lap3 lap4 (ape1).

**Results**

**Biosynthesis and Processing of Aminopeptidase I**

Subsequent to the initial characterization of API as a vacuolar protein, little work has been done to examine the biogenesis of this hydrolase. In part, this may have been due to the identification of API as a glycoprotein (Metz and Röhm, 1976) that was processed in a PEP4-dependent manner (Trumbly and Bradley, 1983); these results suggested that it was delivered to the vacuole by a process that was essentially the same as that used by the other well-studied soluble vacuolar hydrolases. The presence of an amino terminus that bears no resemblance to the standard signal sequence, however, suggested a unique mechanism for ER translocation and/or vacuolar delivery (Chang and Smith, 1989; Cueva et al., 1989).

To examine the biosynthesis of this enzyme more carefully, we generated antisera to API using synthetic peptides based on the deduced amino acid sequence of the API gene. To characterize this antiserum, we constructed a yeast strain deleted for the chromosomal API gene. A strain deleted and disrupted at the API locus, DY101 (Δape1::LEU2), was constructed by standard techniques as described in Materials and Methods (Fig. 1). We prepared soluble protein extracts from strain DY101 and the isogenic wild-type strain. The extracts were electrophoresed under nondenaturing conditions and stained for aminopeptidase activity. Three of the four leucine aminopeptidase activities, corresponding to aminopeptidases I, II, and IV, are easily observed in a wild-type strain (Fig. 2). Only the band corresponding to API is detected in purified vacuoles (data not shown) in agreement with the previously reported localization of this hydrolase (Matile et al., 1971; Frey and Röhm, 1978). Strain DY101 lacks the band representing API activity. When this strain is transformed with a plasmid encoding the API gene, the API activity band is restored. These activity data confirm that we have disrupted the API locus in strain DY101.

We analyzed the peptide-specific antiserum in strain DY101, as well as lap mutant strains derived from the original aminopeptidase mutants of Trumbly and Bradley (1983). The antiserum is able to precipitate two bands from a wild-type strain that are not recognized by preimmune serum (Fig. 3). These bands have an apparent molecular mass of ~50 and 61 kD. The size of the lower molecular mass species is in agreement with the predicted size of mature API based on the deduced amino acid sequence of the API gene and is the same as that reported previously (Metz and Röhm, 1976; Chang and Smith, 1989). The more slowly migrating band runs at a higher molecular mass than is predicted for the precursor form of API (57 kD) based on the deduced amino acid sequence (Chang and Smith, 1989). A 61-kD in vitro translation product for API, however, was previously reported (Distel et al., 1983). The two bands show a time-
A m inopeptid ase I Is Not Glycosylated

All of the soluble vacuolar hydrolases that transit through the secretory pathway undergo some form of glycosyl modification (Klionsky et al., 1990). Core oligosaccharides are added in the ER, and these are subsequently modified and elongated during passage through the Golgi complex. These glycosyl modifications result in molecular weight changes that are easily detectable by SDS-PAGE. CPY, for example, is first detected as the ER glycosylated (pl) form. Subsequent modifications within the Golgi complex generate the higher molecular weight (p2) form of the enzyme (Stevens et al., 1982; Zubenko et al., 1983; Fig. 4). API has four potential sites for the addition of N-linked oligosaccharides. Two of the sites have the less frequently used Asn-X-Ser sequence (Moehle et al., 1987), so it is expected that glycosylation might only occur at two of the four sites. No molecular weight shift is seen with API, however, other than the decrease in molecular weight due to removal of the propeptide (Fig. 4).

The lack of an observable molecular weight shift in the precursor form of API (Fig. 4) coupled with the observation that the product from an in vitro transcription/translation reaction comigrates with the precursor protein synthesized in vivo (Fig. 3) suggests that the protein may not be glycosylated. To examine this further, we tested for the presence of N-linked and O-linked carbohydrates on API through the use of tunicamycin and Con A. Tunicamycin blocks the addition of N-linked carbohydrates to proteins. After synthesis in the presence of tunicamycin, proteins that normally receive N-linked glycosylation migrate as lower molecular weight species during SDS-PAGE relative to the untreated wild-type protein. Con A can be used to test for the presence of either N-linked or O-linked glycosylation because either type of oligosaccharide can bind to this lectin. Both types of analysis indicate that API is not glycosylated.

Yeast cells were labeled with Tran35S-label for 30 min, in the presence or absence of tunicamycin. The cells were then subjected to a nonradioactive chase for 0 or 120 min. Samples were removed at each time point, and half of each sample was analyzed directly (total) by immunoprecipitation and gel electrophoresis. The remaining sample was treated with Con A coupled to sepharose, and separated into Con A precipitable (pellet) and nonprecipitable (supernatant) fractions. CPY contains four sites for N-linked glycosylation (Valls et al., 1987) and normally contains ~10,000 D of carbohydrate (Hasilik and Tanner, 1978b). In the presence of tunicamycin, CPY is not glycosylated and migrates as lower molecular weight species (Hasilik and Tanner, 1978b; Fig. 5). Consistent with its being glycosylated, wild-type CPY is precipitable with Con A. CPY synthesized in the presence of tunicamycin, however, does not bind this lectin and is recovered exclusively in the supernatant fraction (Fig. 5). In contrast, API does not show a decrease in molecular weight when synthesized in the presence of tunicamycin (Fig. 5). This suggests that it does not contain N-linked oligosaccharides. In addition, API does not bind Con A either in the presence or absence of tunicamycin. Because proteins that contain either N-linked or O-linked oligosaccharides are precipitable with Con A, this suggests that API is not glycosylated.

Treatment with tunicamycin is nonspecific, because it blocks the glycosylation of all proteins in the cell. This results in secondary defects and tends to slow the transit of proteins through the secretory pathway (Stevens et al., 1982). This can be seen by comparing the forms of CPY present at specific time points in the presence and absence of tunicamycin (Fig. 5). In the absence of tunicamycin, most of CPY is present as the mature form even at the 0-min
Aminopeptidase I Does Not Transit through the Secretory Pathway

The absence of a consensus signal sequence, the apparent lack of glycosylation, and the relatively long half-time of maturation suggest that API may not transit through the secretory pathway. We addressed the issue of secretory pathway transit by examining the dependence of API processing on Sec proteins. Mutations in various SEC genes result in blocks in transit at different steps of the secretory pathway at the nonpermissive temperature (Esmon et al., 1981; Novick et al., 1981). The sec12 and sec23 mutants are blocked in ER to Golgi transport at the nonpermissive temperature and show a very rapid onset of the secretion defect (Kaiser and Schekman, 1990; Graham and Emr, 1991; Rexach and Schekman, 1991). Yeast sec mutant strains were grown at 23°C, then shifted to a nonpermissive temperature of 37°C for 5 min to inactivate the altered SEC gene product. The cells were then labeled for 5 min and subjected to a nonradioactive chase for 0-120 min at 34°C (Fig. 6). There is no processing of CPY even at the 120-min time point in the sec23 mutant strain and only a small amount of mature CPY in the sec12 mutant. Complete processing is seen in cells labeled and chased at the permissive temperature. In contrast, there is substantial processing of API in both mutant strains at the nonpermissive temperature (Fig. 6). Similar results were seen with sec18 and sec7 mutant cells (data not shown). In sec1 mutant cells, there is also a partial block in API processing, although, in this case, there is complete maturation of CPY (data not shown). Because there is no indication that vacuolar hydrolases would transit through the late secretory pathway, this probably reflects general loss of viability of the sec mutant strains; at the long chase times needed to see complete maturation of API, the sec mutant strains are no longer competent for protein transport. This is similar to the result seen with α-mannosidase (Yoshihisa and Anraku, 1990).

The relative sec-independence of API indicates that the protein does not enter the secretory pathway. This is confirmed by a protease sensitivity analysis. Proteins that enter a membrane-bound intracellular compartment are protected from degradation by exogenous protease after gentle lysis of the plasma membrane. In contrast, cytoplasmic proteins are susceptible to proteolytic degradation under these conditions. API activity is localized to the vacuole but previous studies have not addressed the location of the precursor protein. It is possible that the precursor is located in the vacuole but is processed with very slow kinetics. Because mature API, like other mature vacuolar hydrolases, is resistant to proteolysis, it is not possible to determine the location of the mature enzyme by protease protection studies in a wild type strain. To further assess the location of precursor API, spheroplasts were prepared from strain SEY2101Δpep4, which accumulates only the precursor form of API. Spheroplasts were labeled for 20 min and subjected to a nonradioactive chase for either 2 or 135 min. At each chase point, the samples were gently lysed with DEAE-dextran as described in Materials and Methods.

Chase point. By 120 min, all of the CPY is present as the mature form. After treatment with tunicamycin, a significant amount of precursor CPY remains even after 120 min of chase. In contrast, there is no detectable difference in the kinetics of processing of API in the presence or absence of tunicamycin (Fig. 5).
Figure 5. API does not bind Con A and is insensitive to tunicamycin. Wild-type strain SEY2101 was treated with tunicamycin (final concentration of 20 μg/ml) to inhibit glycosylation 15 min before the addition of Tran 35S-label as indicated. Labeling was allowed to continue for 30 min followed by a 0- or 120-min chase. Samples were TCA precipitated and divided in half. One half was immunoprecipitated immediately with antiserum to API or CPY to give a total sample. The remaining half was precipitated with Con A- sepharose and separated into supernatant (not bound to Con A) and pellet (bound to Con A) fractions as described in Materials and Methods. The separate fractions were then immunoprecipitated with antiserum to API and CPY. The positions of precursor and mature CPY and API and the position of protein standards (molecular weight x 10^3) are shown. T, total sample; S, supernatant from Con A; P, pellet from Con A. A and B show immunoprecipitations of CPY from cells labeled in the absence (A) or presence (B) of tunicamycin. C and D show immunoprecipitations of API from cells labeled in the absence (C) or presence (D) of tunicamycin.

These results indicate that at early time points the API precursor is not within a membrane-enclosed compartment. This suggests that the precursor form of API resides in the cytoplasm and is translocated directly into the vacuole.

Vacuolar Targeting of Aminopeptidase I in vps Mutants

If API remains in the cytoplasm after it is synthesized, it may not be affected by vacuolar protein sorting (vps) mutants that were selected on the basis of secreting vacuolar proteins that transit through the secretory pathway (Bankaitis et al., 1986; Rothman and Stevens, 1986; Robinson et al., 1988; Rothman et al., 1989). We examined the localization and processing of API in several representative vps mutant strains. Wild-type and vps mutant cells were labeled for 20 min and subjected to a 120-min chase. The cultures were separated into cell (pellet) and media (supernatant) fractions after centrifugation, and analyzed by immunoprecipitation and SDS-PAGE (Fig. 8). In general, processing of API is more complete in the vps mutant strains than is seen for CPY and PrA. For example, vps5, vps8, and vps17 show substantial maturation of API even though these mutants are extremely defective for processing CPY (Fig. 8) and PrA (Robinson et al., 1988). Many of the vps mutants, however, accumulate precursor API relative to a wild-type strain. The band that
The precursor form of API does not reside within an intracellular compartment. Spheroplasts were prepared from strain SEY201Δapep4, labeled for 20 min, and subjected to a nonradioactive chase for 2 or 135 min. The spheroplasts were gently lysed with DEAE-dextran and treated with pronase and/or TX-100 as described in Materials and Methods. After TCA precipitation, samples were immunoprecipitated with antiserum to CPY or API. The positions of precursor and mature forms of CPY and API are indicated.

In the absence of detergent, ~70% of CPY remains in the pellet fraction (Fig. 9). The 30% that was recovered in the supernatant fraction indicates that there was some lysis of the ER. In contrast, 85–90% of precursor ALP was recovered in the membrane pellet fraction. Under the same conditions, ~95% of API was present in the supernatant fraction (Fig. 9). Treatment with the detergent saponin released virtually all of the CPY into the supernatant fraction by permeabilizing the ER membrane. As before, almost all of API was also present in this fraction. Saponin does not solubilize membrane proteins, and ALP remained associated with the membrane fraction (Schauer et al., 1985; Klionsky and Emr, 1989). Disruption of the membranes with the nonionic detergent TX-100 resulted in the complete solubilization of all three hydrolases (Fig. 9). These results indicate that API is a soluble protein.

**Translocation of Aminopeptidase I into the Vacuole Utilizes a Saturable Component**

The primary evidence that suggests that vacuolar proteins are delivered to the vacuole by a receptor-mediated process is the observation that overproduction leads to precursor accumulation and missorting to the cell surface (Rothman et al., 1986; Stevens et al., 1986). To examine the effect of overproduction on the localization of vacuolar proteins, cells were transformed with multiple copy (2 μ circle) plasmids that overproduce certain of the soluble hydrolases. Cells harboring plasmids that overproduced either CPY or PrA showed precursor accumulation and secretion of the respective hydrolase (Fig. 10). Synthesis from the plasmid pRN1 results in API levels approximately five times higher than that seen from the chromosome. This level of overproduction has no effect on the processing kinetics of API (Fig. 4). The multiple copy plas-
mid pRC1 results in API levels approximately 15–20-fold above the chromosomal level. Even with this increase in API production, none of the protein is secreted into the extracellular fraction (Fig. 10). This further supports the notion that API does not transit through the secretory pathway, because overproduction is not expected to cause secretion of a non-secretory pathway protein.

If translocation of API involves a limiting component, however, overproduction would be expected to affect the half-time of maturation. We compared the kinetics of processing of API through a pulse-chase analysis of cells harboring single or multiple copy APE1 plasmids. API synthesized from the chromosome or the single copy plasmid pRN1 has a processing half-time of ~45 min (Fig. 4). Overproduction of API from the plasmid pRC1 increases the half-time of processing to ~105 min (Fig. 4). This increased level of API had no effect on the sorting of CPY. This suggests that some component required for translocation of API into the

![Figure 8](image1)

**Figure 8.** The API precursor is not secreted from vps mutant cells. Wild-type (SEY6210) and vps mutant yeast strains were labeled for 20 min and subjected to a nonradioactive chase for 0 or 120 min in the presence of BSA and α2-macroglobulin to reduce nonspecific proteolysis. The cultures were separated into cell (C) and medium (M) fractions by centrifugation and immunoprecipitated with antiserum to CPY and API. The positions of precursor and mature forms of CPY and API are shown.

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![Figure 9](image2)

**Figure 9.** The API precursor is a soluble cytoplasmic protein. Spheroplasts were prepared from strain SEY5187 (sec18), shifted to 37°C for 15 min, then labeled and subjected to a nonradioactive chase for 30 min at 32°C. The spheroplasts were lysed and treated with saponin (0.4%) or TX-100 (0.1%) as indicated. The samples were separated into soluble (supernatant) and membrane-associated (pellet) fractions as described in Materials and Methods. The percent of each protein present in the supernatant fraction is calculated as the ratio of protein present in the supernatant fraction divided by the total present in both the supernatant and pellet fractions combined. □, CPY; ■, API; □, ALP.

![Figure 10](image3)

**Figure 10.** Overproduction of API does not result in its secretion from the cell. Yeast cells were transformed with single or multiple copy plasmids carrying the gene coding for API or multiple copy plasmids carrying the gene coding for either CPY or PrA. The cells were labeled for 30 min and subjected to a nonradioactive chase for 90 min. The samples were separated into cell (C) and medium (M) fractions by centrifugation and immunoprecipitated with antiserum to API, CPY, or PrA. The positions of precursor and mature forms of each hydrolase are shown. The genes (in parentheses) carried by the respective plasmids and the proteins they encode are as follows: (A) pRN1 (APE1), API; (B) pRC1 (APE1), API; (C) pTSY1 (PRC1), CPY; (D) pPA3 (PEP4), PrA.
vacular protein α-mannosidase is thought to translocate into the vacuole from the cytoplasm (Yoshihisa and Anraku, 1990). API and α-mannosidase may use the same component(s) for vacuolar delivery. To gain additional insight into this mechanism of vacuolar import, we examined the effect of overproducing α-mannosidase on the processing of API. Synthesis of α-mannosidase from the multiple copy plasmid pAMI results in an ~20-fold increase in activity (Kuranda and Robbins, 1987). This level of overproduction of α-mannosidase resulted in an increase in the half-time of processing of API to ~65 min, whereas there was no effect on the kinetics of CPY maturation (data not shown). In this case, even though the half-time of processing was significantly increased, all of the precursor API was eventually processed to the mature form.

Discussion

In recent years, considerable attention has focused on the mechanisms used to accurately and efficiently sort proteins to specific organelles within eukaryotic cells. For many reasons, the yeast vacuole has been at the center of much of this research. The vacuole is relatively easy to purify biochemically, contains a large number of marker proteins, and is physiologically important to the cell (reviewed in Klionsky et al., 1990). The targeting process for many vacuolar proteins is a complex one; proteins entering the secretory pathway must be selectively retained at, or transit through, a large number of compartments. The factors involved in the final sorting decision that appears to occur at the trans Golgi network (Griffiths and Simons, 1986; Pfeffer and Rothman, 1987) remain largely undefined. At this time, there is no definitive evidence that vacuolar proteins utilize protein receptors in the delivery process. Similarly, even though a number of genes encoding vacuolar proteins have been cloned and sequenced, little progress has been made in identifying the specific nature of vacuolar sorting signals.

Current evidence suggests that soluble and membrane vacuolar proteins that transit through the secretory pathway may use different sorting components (Banta et al., 1988; Robinson et al., 1988; Klionsky and Emr, 1989, 1990; Herman et al., 1991). The precise mechanisms of sorting may also differ with respect to these two different classes of proteins. Soluble proteins are dependent on compartment acidification for efficient vacuolar delivery, whereas membrane proteins are localized to the vacuole relatively independent of changes in lumenal pH (Banta et al., 1988; Klionsky et al., 1992). Analyses of α-mannosidase indicate that some resident proteins may get to the vacuole by a completely different mechanism. This protein appears to enter the vacuole directly from the cytoplasm (Yoshihisa and Anraku, 1990). Other proteins may be translocated directly into the vacuole as part of catabolite inactivation (Funaguma et al., 1985; Chiang and Schekman, 1991). This may be similar to the lysosomal import of certain cytosolic proteins destined for degradation (Dice, 1987; Chiang and Dice, 1988). At present, there are no examples of resident lysosomal proteins that enter this organelle in an analogous manner.

Many differences exist regarding the specific modifications that occur on vacuolar proteins (Klionsky et al., 1990). A careful analysis makes it clear that no single protein can serve as a comprehensive model for vacuolar protein biogenesis; there are significant variations with regard to proteolytic processing and glycosylation. Nonetheless, certain vacuolar proteins have been instrumental in the elucidation of the transport process. CPY has served as a useful model of a soluble vacuolar protein that transits through the secretory pathway (Stevens et al., 1982; Bankaitis et al., 1986; Rothman and Stevens, 1986; Johnson et al., 1987; Valls et al., 1987). Similarly, alkaline phosphatase has many characteristics that make it a useful model for vacuolar membrane proteins (Klionsky and Emr, 1989). The key features of a useful marker protein for following protein sorting are that it undergoes glycosylation and/or proteolytic processing. These processes allow a rapid and specific determination of the location of the protein within the cell. In particular, glycosyl modifications allow an assessment of a protein's position within the secretory pathway, whereas proteolytic removal of a propeptide is a convenient means to kinetically follow vacuolar delivery.

Similarly, to understand the mechanism involved in an alternative, nonsecretory pathway, vacuolar targeting process, it is necessary to have useful marker proteins. At present, only two resident vacuolar proteins have been shown to use this alternative pathway: α-mannosidase and API. For various reasons, α-mannosidase is not likely to be a useful marker protein. The half-time of processing of API is significantly longer than that of secretory pathway mediated vacuolar protein delivery, but it occurs within a time frame that is technically useful. In contrast, the half-time of processing for α-mannosidase is ~10 h (Yoshihisa and Anraku, 1990), making a kinetic analysis of vacuolar delivery difficult to follow. In addition, α-mannosidase is not expressed well unless cells are heat shocked at 37°C (Yoshihisa and Anraku, 1990). Analyses of mRNA levels indicate that API synthesis increases as cells approach stationary phase and when the glucose concentration decreases (Distel et al., 1983; Cueva et al., 1989). The protein is easily detected, however, during logarithmic growth. Because of these characteristics, API will be a useful marker protein to follow vacuolar delivery through this alternate mechanism.

The lack of a consensus signal sequence in API makes it unclear how this protein would enter the ER. The initial characterization of API suggested that it is a glycoprotein (Metz and Rohn, 1976) but the present studies indicate that this is not correct. Several observations suggest that this protein does not in fact enter the secretory pathway: (1) the relatively long half-time for processing of API; (2) the lack of glycosylation; (3) Sec protein independence; (4) lack of secretion from vps mutants or upon overproduction. Precursor API appears to remain in the cytoplasm after synthesis.

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It is sensitive to exogenous protease under conditions where ER and Golgi precursor forms of CPY are protected. These results suggest that API first enters a membrane-enclosed compartment posttranslationally.

Processing of API is relatively independent of the SEC gene products. Precursor API is not completely processed at the nonpermissive temperature, however, in any of the sec mutant strains examined. We believe this reflects the relatively long half-time required for API processing coupled with a reduced viability of sec mutant strains under nonpermissive conditions. Most of the sec mutant strains display a reversible temperature-dependent phenotype; transit of accumulated proteins proceeds after return to a permissive temperature (Novick et al., 1980). The precursor API that accumulated in the sec mutant strains at the restrictive temperature was not processed, however, even after an additional 2-h chase following a return to the permissive temperature (data not shown). In addition, API shows a level of processing in a sec1 mutant strain similar to that seen in the sec12 and sec23 mutants. It is unlikely that delivery of API to the vacuole is dependent on components of the late secretory pathway such as the Sec1 protein. The partial block in API processing exhibited by all of the sec mutant strains probably reflects cell inviability of these strains resulting from extended exposure to elevated temperatures. This indicates a limitation in the use of the sec mutant strains when examining proteins such as API that have long half-times for processing.

Although API does not appear to be sensitive to sec mutations, it is affected by vps mutants. The accumulation of precursor API in vps mutants suggests four possibilities: (1) API may share some sorting components encoded by certain VPS genes that are used by vacuolar proteins that transit through the secretory pathway. (2) The vacuole in many vps mutant strains may not be a competent target for vacuolar delivery. In particular, the class C vps mutants, such as vps16 in Fig. 8, lack a detectable vacuole (Banta et al., 1988) so it is not surprising that API cannot be processed. These mutants also affect a-mannosidase, an observation that is consistent with these two proteins using the same import pathway (Banta et al., 1988). (3) Severe vps missorting phenotypes also result in reduced levels of processing enzymes being localized to the vacuole. This may lower the processing capacity of the organelle such that precursor API located within the vacuole may not be proteolytically matured. (4) API may use a receptor/translocator protein or some other component that is delivered to the vacuole through the secretory pathway. In vps mutants, delivery of this protein may be defective, resulting in inefficient import of API into the vacuole. This would be similar to the proposed sec-dependent delivery of a vacuolar import protein required for translocation of fructose 1,6-bisphosphatase (Chiang and Schekman, 1991). If a receptor/translocator protein is used, it may be saturable. Because API is not in the secretory pathway, it will not be secreted upon saturation of this component. Saturation would be expected to slow the half-time of processing, and this is seen upon overproduction of API from a multiple copy plasmid.

A substantial amount of the API precursor is never matured under conditions where it is overproduced. This may indicate that some level of the precursor is missorted. A more likely explanation for the lack of complete maturation upon overproduction is that one or more cytoplasmic components such as an hsp70 protein (Chirico et al., 1988; Deshaies et al., 1988) are required for maintaining the precursor in a translocation competent state. Overproduction titrates out this component, causing the remaining precursor to prematurely fold into a form that is no longer compatible to interact with the translocation machinery or is no longer competent to cross a membrane. This may be similar to the model proposed for the E. coli maltose binding protein (MBP) (Randall and Hardy, 1986). If translocation of pre-MBP does not occur within a specific time-frame, the precursor protein folds into a conformation that is not longer competent for export. Folding of pre-MBP and maintenance of the translocation competent form may be modulated through interaction with the molecular chaperone SecB (Kumamoto, 1991).

Chaperonins or polypeptide chain binding proteins (Ellis, 1987; Rothman, 1989) are involved in allowing proteins to translocate across membranes by maintaining them in a partly unfolded state. In addition to some bacterial secreted proteins, this has been suggested for certain eukaryotic proteins that enter mitochondria, chloroplasts, and the ER (reviewed in Ellis and van der Vies, 1991; Kumamoto, 1991; Zeilstra-Ryalls et al., 1991). Accessory proteins may also be needed for protein refolding after translocation is complete. The GRP78/BiP protein located within the ER lumen and the mitochondrial hsp60 protein, for example, have been implicated in the folding of translocated proteins (Bole et al., 1986; Munro and Pelham, 1986; Ostermann et al., 1989). These molecular chaperones are often members of the heat shock family of stress proteins (Schlesinger, 1990; Ellis and van der Vies, 1991). Heat shock proteins have been shown to be involved in the translocation of vacuolar proteins into the ER lumen (Deshaies et al., 1988). A member of the heat shock protein family has not been shown to play a role in the transport of vacuolar proteins directly into the vacuole, although an hsp70 protein has been implicated in the import of proteins destined for lysosomal degradation (Chiang et al., 1989). We are currently addressing the question of whether API utilizes a molecular chaperone for translocation into the vacuole. It is interesting to consider the possibility that API also requires some type of binding protein within the vacuolar lumen to promote its proper folding after translocation.

The observation that overproduction of a-mannosidase also increases the half-time of processing of API suggests that both proteins may use a common component in the translocation process. The mechanisms of vacuolar import of the resident protein a-mannosidase or a protein destined for degradation such as fructose 1,6-bisphosphatase have not been elucidated. The entry of these proteins into the vacuole is difficult to follow because of the long half-times of processing and the lack of a specific cleavage event, respectively. An analysis of API translocation may allow a detailed dissection of this alternate pathway. Certain features of the unique API amino terminus may be critical in allowing the translocation event to proceed. We are currently investigating the location of the sorting signal of API. In addition, we have begun an in vitro analysis of API translocation into the vacuole in order to define trans-acting components required for vacuolar localization of this hydrolase.

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