

Aminopeptidase I of *Saccharomyces cerevisiae* Is Localized to the Vacuole Independent of the Secretory Pathway

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Abstract. The *Saccharomyces cerevisiae* *APE1* 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 pro-

peptide occurs in a *sec*-independent manner. Neither the precursor nor mature form of API are secreted into the extracellular fraction in *vps* 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)¹ appears to be localized to the vacuole based on subcellular fractionation (Matile et al., 1971; Frey and Röhms, 1978). Initial studies of API characterized it as being a glycoprotein containing 12% carbohydrate (Metz

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1. *Abbreviations used in this paper:* API, aminopeptidase I; TX-100, Triton X-100; YNB, yeast nitrogen base; YPD, 1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose.

and Röhm, 1976). 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, *APE1* (previously designated *LAP4*), has been cloned and the nucleotide sequence determined (Cueva et al., 1989; Chang and Smith, 1989). The *APE1* 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 $\sim 600,000$ 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 $\sim 45,000$ 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; Sherman et al., 1979), modified Wickerham's minimal medium containing 0.2% yeast extract (Robinson et al., 1988), and yeast nitrogen base (YNB) medium with the appropriate supplements were used for growing yeast.

Reagents

Lyticase was obtained from Enzogenetics (Corvallis, OR), Tran³⁵S-label was from ICN Radiochemicals (Irvine, CA), DNA restriction and modifying enzymes were from New England Biolabs, Inc. (Beverly, MA), α_2 -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 Biotech (Madison, WI). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Wheat germ lysate was generously supplied by William Etinger 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 antiserum to API, we had two synthetic peptides made (Multiple Peptide Systems, San Diego, CA) based on the deduced amino acid sequence of the *APE1* 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 antiserum in New Zealand White rabbits.

Table I. Yeast Strains

Yeast strains	Genotype	Source
BJ3044	MATa can1 lys2-801 prb1- Δ 1.6 ura3-52	Moehle et al., 1989
DBY746	MAT α leu2-3,112 his31 ura3-52 trp1-289	Yeast Genetic Stock Center
DYY101	MAT α leu2-3,112 ura3-52 his3- Δ 200 trp1- Δ 901 ade2-101 suc2- Δ 9 GAL ape1::LEU2	This study
NAYII-17	MAT α lap1 lap2 lap3 lap4 leu2-3,112 his3- Δ 1 ura3-52 trp1-289	N. Garcia-Alvarez (University of Oviedo)
NAYIII-1	MAT α lap1 lap2 lap3 leu2-3,112 his3- Δ 1 ura3-52	N. Garcia-Alvarez
SEY2101	MATa leu2-3,112 ura3-52 ade2-1 suc2- Δ 9	Emr et al., 1983
SEY2101 Δ pep4	MATa leu2-3,112 ura3-52 ade2-1 suc2- Δ 9 Δ pep4::LEU2	Klionsky et al., 1988
SEY5187	MATa sec18-1 leu2-3,112 ura3-52 suc2- Δ 9	Klionsky et al., 1988
SEY6210	MAT α leu2-3,112 ura3-52 his3- Δ 200 trp1- Δ 901 lys2-801 suc2- Δ 9 GAL	Robinson et al., 1988
SF274-3A	MAT α gal2 sec12-4	C. Field and R. Schekman (University of California, Berkeley)
SF309-2C	MAT α gal2 sec23-1	C. Field and R. Schekman

Plasmid Construction

The *APE1* gene was cloned by complementation of the *lap4* mutation as described previously (Cueva et al., 1989). One of the multicopy number (2μ circle) complementing plasmids, pRC1, contains a 4.75-kb BamHI fragment encoding *APE1*, cloned into the plasmid YEpl3 (Nasmyth and Tatchell, 1980). The centromeric (CEN4 ARS1) plasmid pRN1 was constructed by cloning a 4.5-kb SphI fragment encoding the entire *APE1* gene from pRC1 into the SphI site of YCp50 (Johnston and Davis, 1984). To carry out in vitro transcription/translation of *APE1*, a 1.69-kb DNA fragment containing the *APE1* gene was amplified using the polymerase chain reaction. Oligonucleotides containing a Sall site before nucleotide -3 and a HindIII site starting at nucleotide 1685 of the *APE1* gene (Cueva et al., 1989) were used for the reaction. The resulting fragment was ligated into pKK4 that had been restricted with Sall 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 pSP65 (Melton et al., 1984) in which the SphI 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 pTSY1 (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 *APE1* locus, the *APE1* 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 pRC1 encoding a COOH-terminal portion of API was cloned into the pUC19 derivative. The resulting plasmid, pDSY1, was restricted with BamHI. Second, a 2.0-kb BamHI fragment from pRC1 was ligated into the restricted BamHI site of pDSY1 to generate plasmid pDSY2 (Fig. 1). This plasmid, containing the entire *APE1* gene, was restricted with EcoRI to remove almost the entire *APE1* coding region, and the overhanging ends were blunted with the Klenow fragment of DNA polymerase I. A XhoI/Sall fragment containing the *LEU2* gene from the plasmid YEpl3 (Nasmyth and Tatchell, 1980) was ligated into the blunt-ended EcoRI site of pDSY2 to generate the *APE1* disruption plasmid pDSY3 (Fig. 1). This plasmid was linearized with BamHI and Sall and used to transform yeast strain SEY6210 to generate the chromosomal *APE1* 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 non-radioactive 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 μ g/ml final concentration) for 15 min prior to the addition of label. Cells were labeled for 30 min and then subjected to a non-radioactive chase for 0 or 120 min. At the end of the chase, cells were precipitated with TCA, washed with acetone, dried, and resuspended in 100 μ l 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 μ l 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 antiserum to API and CPY (total sample). The remaining half was diluted with 1 ml Con A 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 μ l 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 pellet from the Con A precipitations was washed twice with Con A reaction buffer and boiled in 100 μ l MURB. The pellet samples were then diluted with 1 ml

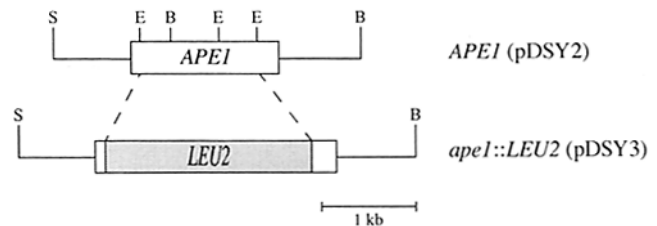


Figure 1. Disruption of the *APE1* locus. A restriction enzyme map is shown of the ~ 3.3 -kb genomic DNA fragment of *APE1* present in plasmid pDSY2. The EcoRI fragment from within the *APE1* coding region was removed, and the ends were blunted. An ~ 2.2 -kb XhoI/Sall fragment encoding the yeast *LEU2* gene was blunt-ended and inserted into the deleted *APE1* gene to generate plasmid pDSY3. The plasmid pDSY3 was used to disrupt the chromosomal *APE1* locus as described in Materials and Methods. The white box represents the *APE1* gene, and the stippled box represents the *LEU2* coding region. Restriction enzymes are as follows: B, BamHI; E, EcoRI; S, Sall.

Con 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 $\Delta pep4$. Spheroplasts were labeled for 20 min and subjected to a 2-min (precursor API) or 135-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% ficoll and gently lysed by the addition of DEAE-dextran (Dürr 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 μ g/ml) or pronase and TX-100 (0.2%). The samples were incubated at 0°C for 30 min followed by 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 μ g/ml α_2 -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 antiserum to CPY, API, and ALP.

In Vitro Synthesis of API

In vitro transcription of the *APE1* gene was carried out using the pKK4/*APE1* construct described above. The transcription reaction was carried out essentially as described (Melton et al., 1984) using 5 μ g 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.

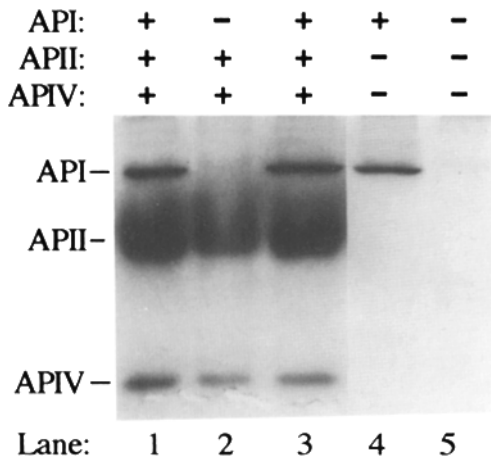


Figure 2. Leucine aminopeptidase activity in wild-type and mutant strains. Soluble protein extracts were prepared from strains containing the indicated aminopeptidases. The extracts were electrophoresed under nondenaturing conditions and stained for activity using L-leucine β -naphthylamide as the substrate as described in Materials and Methods. The strains and their relevant genotypes are as follows: (lane 1) SEY6210, *APE1*; (lane 2) DYY101, Δ *ape1*; (lane 3) DYY101/pRN1, Δ *ape1/APE1*; (lane 4) NAYIII-1, *lap1 lap2* (*APE1*); (lane 5) NAYII-17, *lap1 lap2 lap3 lap4* (*apel*).

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 antiserum to API using synthetic peptides based on the deduced amino acid sequence of the *APE1* gene. To characterize this antiserum, we constructed a yeast strain deleted for the chromosomal *APE1* gene. A strain deleted and disrupted at the *APE1* locus, DYY101 (Δ *ape1::LEU2*), was constructed by standard techniques as described in Materials and Methods (Fig. 1). We prepared soluble protein extracts from strain DYY101 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 DYY101 lacks the band representing API activity. When this strain is transformed with a plasmid encoding the *APE1* gene, the API activity band is restored. These activity data confirm that we have disrupted the *APE1* locus in strain DYY101.

We analyzed the peptide-specific antiserum in strain DYY101, 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 \sim 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 *APE1* 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-

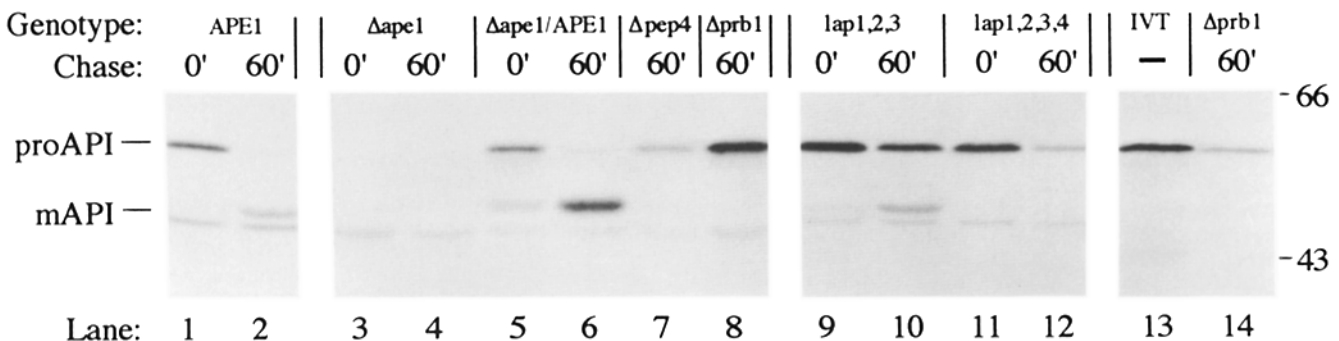


Figure 3. Immunoprecipitation of API from wild-type and mutant yeast strains and in vitro transcription/translation reactions. Complete genotypes of the strains are listed in Table I. Cells were labeled for 40 min followed by a nonradioactive chase for 0 or 60 min as indicated. Radiolabeled cell extracts were immunoprecipitated with antiserum to API as described in Materials and Methods. The in vitro transcription and translation reactions were performed as described in Materials and Methods. The positions of precursor and mature API and the position of protein standards (molecular weight $\times 10^3$) are shown. Immunoprecipitation with preimmune serum gave identical results to that shown for strain DYY101 (lanes 3 and 4). The band that runs just below mature API is due to nonspecific cross-reacting material. The strains used, the relevant phenotypes, and the corresponding lane numbers are as follows: (lanes 1 and 2) SEY6210, *API*⁺; (lanes 3 and 4) DYY101, *API*⁻; (lanes 5 and 6) DYY101/pRN1, *API*⁻/*API*⁺; (lane 7) SEY2101 Δ *pep4*, *API*⁺ PrA⁻; (lane 8) BJ3044, *API*⁺ PrB⁻; (lanes 9 and 10) NAYIII-1, *API*⁺ *APII-IV*⁻; (lanes 11 and 12) NAYII-17, *API-IV*⁻; (lane 13) in vitro *API*; (lane 14) BJ3044, *API*⁺ PrB⁻.

dependent precursor-product relationship. In addition, only the higher molecular mass form is seen in a *pep4* mutant strain (Fig. 3). This is consistent with the known dependence of API activity on the *PEP4* gene (Trumbly and Bradley, 1983) and indicates that this slowly migrating band is the API precursor. The additional molecular mass does not seem to be accounted for by carbohydrate (see below), so we suggest a slightly aberrant migration.

Neither of these bands is seen in strain DYY101, that is disrupted at the chromosomal *APE1* locus (Fig. 3). When strain DYY101 is transformed with a plasmid encoding the *APE1* gene, both bands are again detected. In addition, when API is immunoprecipitated from a strain transformed with a multiple copy (2μ circle) plasmid encoding *APE1*, higher levels of API are detected (Fig. 4). These results suggest that the antiserum specifically recognizes the products of the *APE1* gene. The API immunoprecipitated from a *prb1* mutant strain migrates at the position of the API precursor seen in a *pep4* mutant strain (Fig. 3). This suggests that PrB is the enzyme that is directly involved in the proteolytic maturation of API; the absence of API activity in a *pep4* mutant strain is due to the block in PrB maturation (Hemmings et al., 1981; Moehle et al., 1989) and not the absence of PrA per se.

Yeast strain NAYII-17 lacks the four characterized leucine aminopeptidase activities (Trumbly and Bradley, 1983; Fig. 2). In agreement with this, we detect no mature API in this strain by radiolabeling and immunoprecipitation (Fig. 3). The API precursor is seen in strain NAYII-17, but it appears to be slowly degraded without being converted into the mature form. The *APE1* mutation in this strain has not been characterized and may be a missense mutation that renders the protein inactive and unstable. Strain NAYIII-1 is similar to strain NAYII-17 in lacking all of the leucine aminopeptidase activities except for API (Fig. 2). As expected, an immunoprecipitation of this strain reveals the presence of both API bands (Fig. 3). This further confirms that we are specifically detecting API and not the other leucine aminopeptidases present in *S. cerevisiae*. Finally, the antiserum specifically immunoprecipitates the primary product from an in vitro transcription/translation reaction carried out using mRNA prepared from a construct containing the *APE1* gene (Fig. 3).

In a wild-type strain, there is a time-dependent appearance of the lower molecular weight API species concomitant with the disappearance of the higher molecular weight form, suggesting a precursor-product relationship (Fig. 3). To better characterize this relationship, we examined the processing kinetics of API. Pulse-chase labeling of wild-type yeast cells indicates that API is processed to the mature form with a half-time of approximately 45 min (Fig. 4). The same result was seen with wild-type cells containing a single-copy plasmid encoding *APE1*. This is in contrast to the processing half-time of ~ 6 min seen for CPY (Fig. 4), PrA, or ALP (Hasilik and Tanner, 1978a; Klionsky et al., 1988; Klionsky and Emr, 1989).

Aminopeptidase 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 (p1) 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 oligosaccharides 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 Tran^{35}S -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 $\sim 10,000$ 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

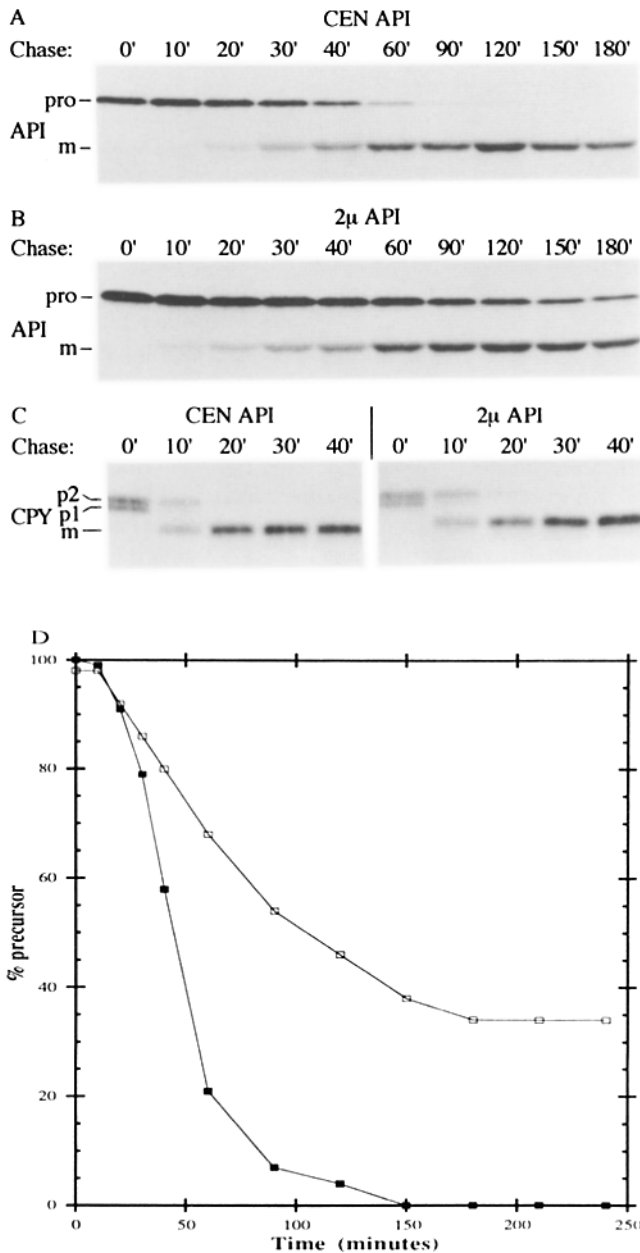


Figure 4. Processing kinetics of API. Strain SEY6210 harboring either the single copy plasmid pRN1 (*A*) or the multiple copy (2μ) plasmid pRC1 (*B*) was labeled for 10 min and subjected to a non-radioactive chase. Aliquots were removed at the indicated times (min) and precipitated with TCA. The samples were immunoprecipitated with antiserum to API (*A* and *B*) or CPY (*C*) as described in Materials and Methods. The positions of precursor and mature forms of API and CPY are indicated. Essentially identical results were obtained for strain SEY6210 and strain SEY6210 transformed with the plasmid pRN1. (*D*) Graphical analysis of the autoradiograms from *A* and *B*. The closed boxes correspond to SEY6210/pRN1 (*CEN* API), and the open boxes to SEY6210/pRC1 (2μ API). The percent precursor values are calculated as precursor divided by precursor plus mature API at each time point.

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).

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 SEY2101 Δ *pep4*, which accumulates only the precursor form of API. Spheroplasts were labeled for 20 min and subjected to a non-radioactive 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.

Lysis with DEAE-dextran preserves the integrity of the ER and Golgi complex as well as that of the vacuole (Klionsky and Emr, 1989). The lysed samples were divided into three aliquots and treated with pronase in the presence and absence of detergent (Fig. 7). At the 2-min chase point, both the p1 and p2 forms of CPY are present. These precursors

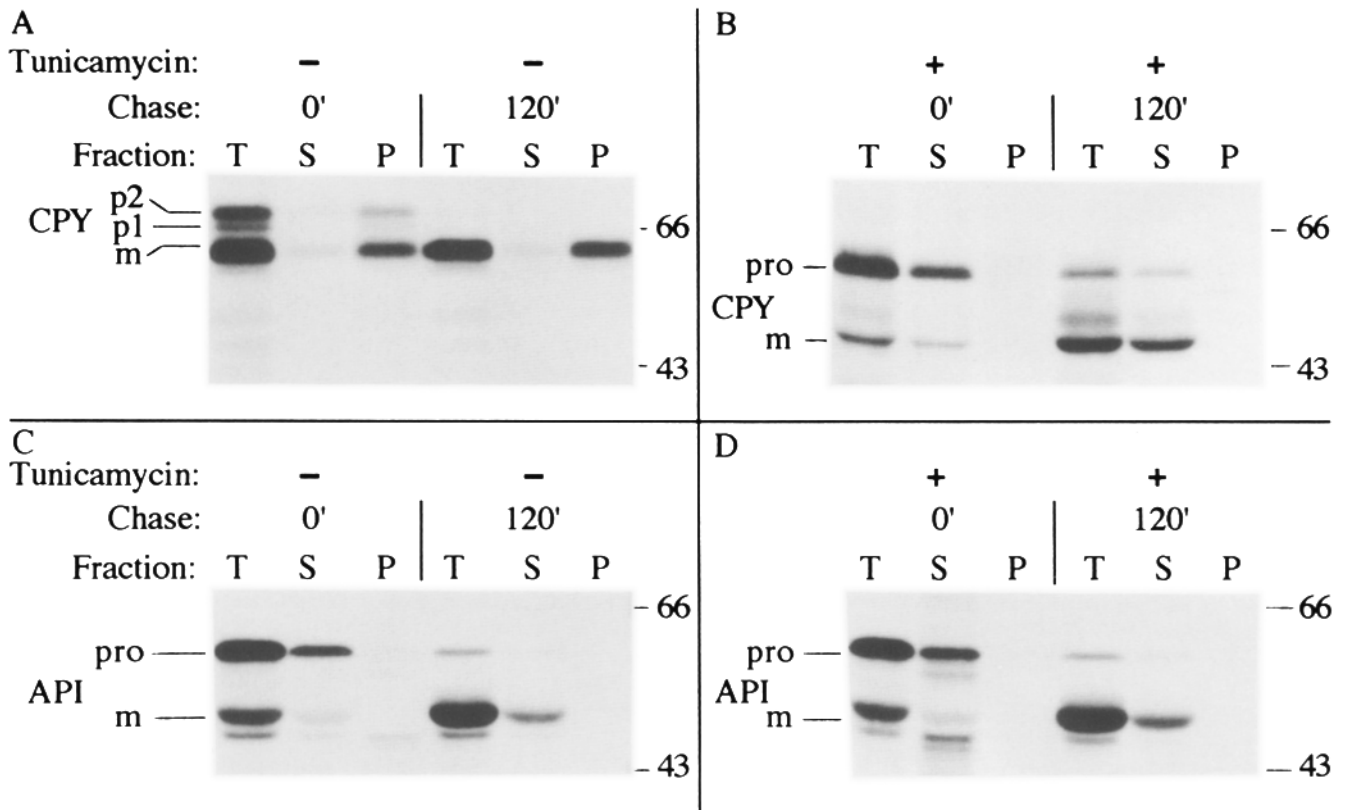


Figure 5. API does not bind Con A and is insensitive to tunicamycin. Wild-type strain SEY2101 was treated with tunicamycin (final concentration of 20 $\mu\text{g/ml}$) to inhibit glycosylation 15 min before the addition of Tran ^{35}S -label as indicated. Labeling was allowed to continue for 30 min followed 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 $\times 10^3$) 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.

sors are resistant to exogenous protease in the absence of detergent, indicating that they are protected within the ER and Golgi. At the 135-min chase point, all of the CPY would be expected to have entered the vacuole and is seen as the p2 form because the vacuoles lack processing activity. This p2 precursor is also protected from exogenous protease in the absence of detergent indicating that the vacuoles are intact. In contrast, the API precursor present after a 20-min label and 2-min chase is almost completely degraded to the size of mature API by added protease in the presence or absence of detergent. This indicates it is not inside a membrane-bound compartment, such as the vacuole. In contrast, at the 135-min time point essentially all of the API precursor is now in a compartment, presumably the vacuole, that protects it from exogenous protease.

The small amount of resistant API precursor seen at the 2-min chase point most likely represents incomplete lysis of the spheroplasts by DEAE-dextran. This is supported by analyzing the susceptibility of ALP to exogenous protease. The cytoplasmic portion of ALP is accessible to exogenous protease in lysed spheroplasts when the intracellular organelles are intact (Klionsky and Emr, 1989). Approximately 20% of ALP remained insensitive to protease at the 2-min chase point (data not shown). This implies that one-fifth of the spheroplasts did not lyse under these conditions.

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

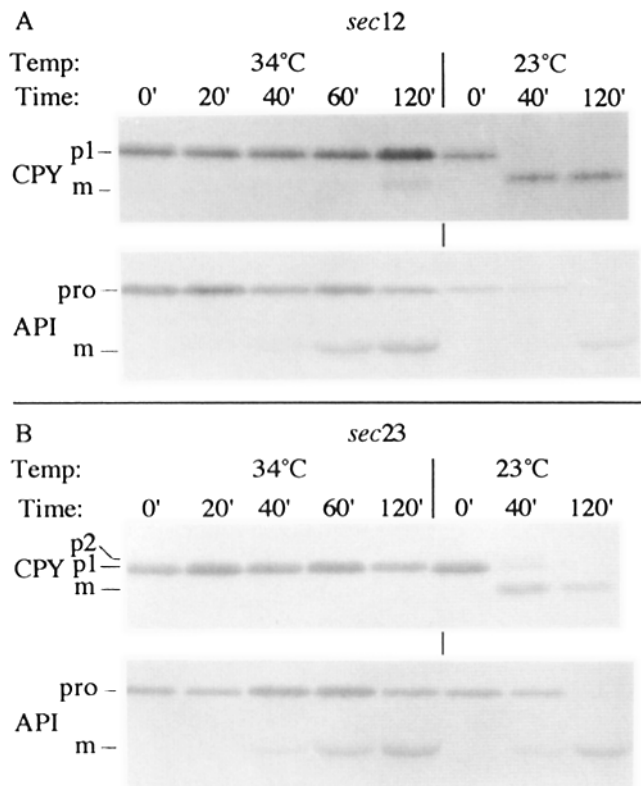


Figure 6. Processing of aminopeptidase I is *sec*-independent. Temperature sensitive *sec12* (A) or *sec23* (B) mutant strains were grown to mid-log phase at 23°C, and half of each culture was shifted to 37°C for 5 min. Both strains were labeled for 5 min at 23°C or 34°C as indicated, and then chase was initiated by the addition of methionine/cysteine as described in Materials and Methods. Samples were removed at the indicated times (min), TCA precipitated, and then immunoprecipitated with antiserum to CPY or API. Essentially identical results as those shown for the *sec23* mutant strain were seen with a *sec1* mutant strain. The positions of precursor and mature forms of CPY and API are shown.

runs below precursor API seen in certain of the *vps* mutant strains is a degradation product (data not shown). It is not known if this species represents an intermediate in the normal processing pathway.

The *vps* mutant defects result in the secretion of the soluble vacuolar hydrolases CPY (Fig. 8), PrB, and PrA (data not shown; Robinson et al., 1988), as seen by the presence of precursor forms in the medium fraction. API is not seen in the extracellular fraction, however, from any of the *vps* mutants. The absence of API from the media fractions is only meaningful if it is a soluble protein; membrane proteins would always remain associated with the cell pellet. API is reported to be a soluble hydrolase, but the apparent discrepancy with regard to glycosylation indicated that further studies with regard to solubility were necessary. To simplify this analysis, we prepared spheroplasts from *sec18* mutant cells (blocked in ER to Golgi transit) and labeled them at the nonpermissive temperature. This resulted in the production of only the ER form of CPY and the vacuolar membrane protein ALP. After labeling and chase, the spheroplasts were osmotically lysed and incubated with saponin or TX-100. The samples were separated into supernatant and pellet fractions by centrifugation and analyzed by immunoprecipitation.

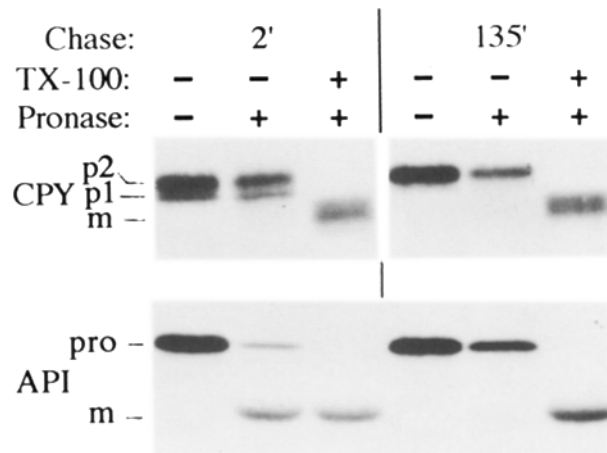


Figure 7. The precursor form of API does not reside within an intracellular compartment. Spheroplasts were prepared from strain SEY2101 Δ *pep4*, labeled for 20 min, and subjected to a nonradioactive chase for 2 or 135 min. The spheroplasts were gently lysed with DEAE-dextran and treated with pronase 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 (2μ circle) plasmids that overproduce certain of the soluble hydrolases. Cells were labeled for 30 min followed by a 90-min chase. The cultures were separated into cell and medium fractions, and analyzed by immunoprecipitation. 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-

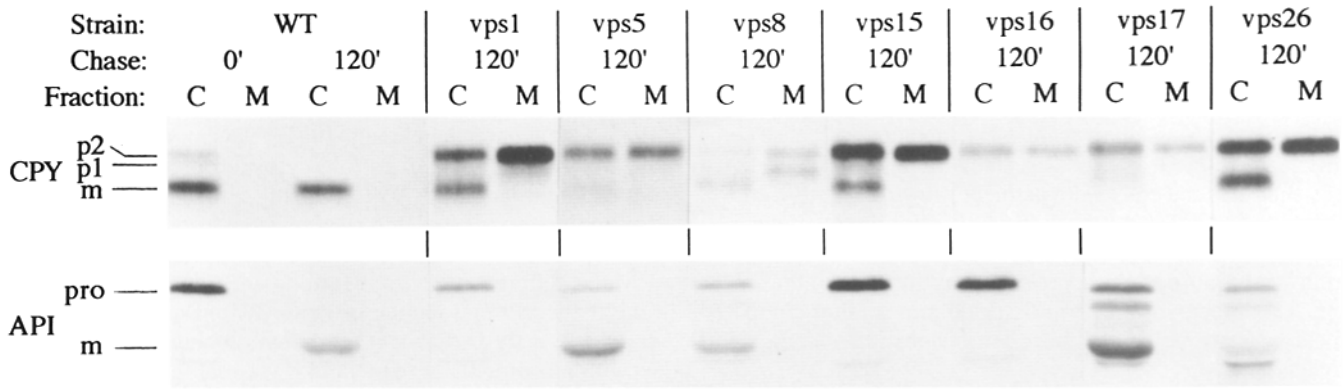


Figure 8. The API precursor is not secreted from *vps* mutant cells. Wild-type (SEY6210) and *vps* mutant yeast strains were labeled for 20 min and subjected to a nonradioactive chase for 0 or 120 min in the presence of BSA and α_2 -macroglobulin to reduce nonspecific proteolysis. The cultures were separated 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.

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 pro-

cessing 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

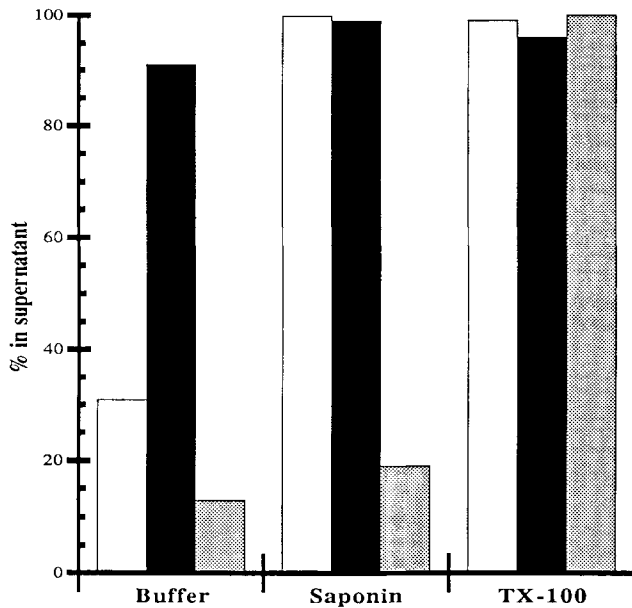


Figure 9. The API precursor is a soluble cytoplasmic protein. Spheroplasts were prepared from strain SEY5187 (*sec18*), shifted to 37°C for 15 min, then labeled and subjected to a nonradioactive chase for 30 min at 32°C. The spheroplasts were lysed and treated with saponin (0.4%) or TX-100 (0.1%) as 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.

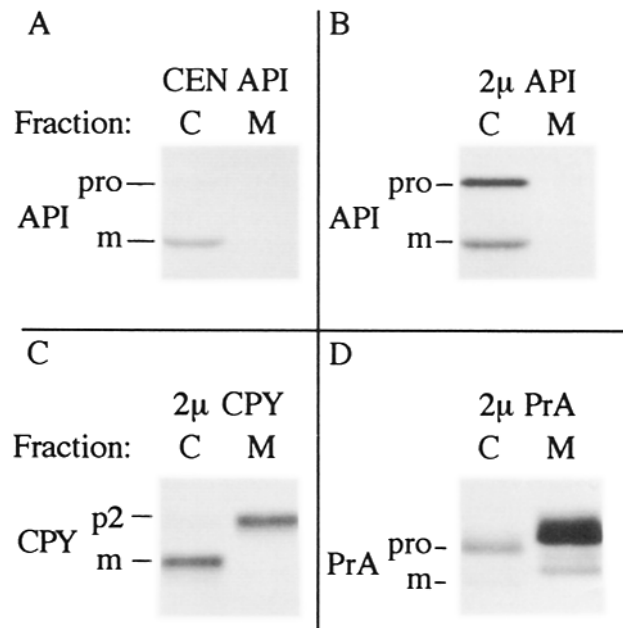


Figure 10. Overproduction of API does not result in its secretion from the cell. Yeast cells were transformed with single or multiple copy plasmids carrying the gene coding for API or multiple copy plasmids carrying the gene coding for either CPY or PrA. The cells were labeled for 30 min and subjected 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.

vacuole is saturable. Alternatively, the increased half-time could indicate that the processing reaction itself is rate-limiting. Approximately 30–35% of the API synthesized from pRC1, however, remains in the precursor form even after extremely long chase points. The labeling and chase conditions were carried out as described in Materials and Methods such that the cells were actively growing over the entire chase period (data not shown). Cell death then cannot account for the lack of processing. The precursor that remains after 4 h or more of chase probably has not entered the vacuole. This precursor may be missorted into another compartment or may be folded into a translocation incompetent form.

The vacuolar 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 pAM1 results in an \sim 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 \sim 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 also 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 luminal 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 undergo glycosyl modification 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 \sim 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 Röhm, 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.

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* mutants 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 α -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 compo-

nents 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 no 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 α -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 α -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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