Abstract. Although the majority of known vacuolar proteins transit through the secretory pathway, two vacuole-resident proteins have been identified that reach this organelle by an alternate pathway. These polypeptides are targeted to the vacuole directly from the cytoplasm by a novel import mechanism. The best characterized protein that uses this pathway is aminopeptidase I (API). API is synthesized as a cytoplasmic precursor containing an amino-terminal propeptide that is cleaved off when the protein reaches the vacuole. To dissect the biochemistry of this pathway, we have reconstituted the targeting of API in vitro in a permeabilized cell system. Based on several criteria, the in vitro import assay faithfully reconstitutes the in vivo reaction. After incubation under import conditions, API is processed by a vacuolar-resident protease, copurifies with a vacuole-enriched fraction, and becomes inaccessible to the cytoplasm. These observations demonstrate that API has passed from the cytoplasm to the vacuole. The reconstituted import process is dependent on time, temperature, and energy. ATP\textsubscript{S} inhibits this reaction, indicating that API transport is ATP driven. API import is also inhibited by GTP\textsubscript{S}, suggesting that this process may be mediated by a GTP-binding protein. In addition, in vitro import requires a functional vacuolar ATPase; import is inhibited both in the presence of the specific V-ATPase inhibitor baflomycin A\textsubscript{1}, and in a yeast strain in which one of the genes encoding a V-ATPase subunit has been disrupted.

A defining feature of eukaryotic cells is the compartmentalization of essential cellular functions in membrane-bound organelles. This segregation necessitates correct and efficient delivery of polypeptides from their site of synthesis to their site of action. Proteins destined for organelles contain signals that allow them to be recognized by the correct sorting machinery and directed to their resident location (Verner and Schatz, 1988). The mechanisms involved in protein sorting reactions can be divided into several major classes. Mitochondrial protein import exemplifies the direct membrane translocation mechanism, whereby the translocating polypeptide is carried from the cytosol, across the lipid bilayer into the lumen of the organelle (Hannavy et al., 1993). In secretory pathway transport, the translocating polypeptide first crosses the ER membrane, and is then passed through the remainder of the compartments inside membrane-bound vesicles (Pryer et al., 1992). Endocytosis and autophagy represent a third mechanism (Seglen and Bohley, 1992; Riezman, 1993). Proteins taken up by these pathways are enclosed in membrane-bound vesicles straight-away, bypassing any direct membrane translocation steps. Each mechanism of translocation uses a distinct set of proteinaceous machinery. In addition, lipid composition also plays a part in protein translocation as alterations in phospholipid or cholesterol content can alter protein transport through the secretory pathway (Bankaitis et al., 1990; Bretschler and Munro, 1993).

The yeast vacuole performs a myriad of cellular functions including osmoregulation, metabolite storage, and protein turnover (Klionsky et al., 1990). Most resident proteins reach this organelle via the secretory pathway (Stack and Emr, 1993), while proteins destined for degradation are delivered from the cell surface through the endocytic pathway (Riezman, 1985, 1993), or from the cytoplasm by autophagy (Tsukada and Ohsumi, 1993; Baba et al., 1994; Thumm et al., 1994). In contrast to the majority of characterized vacuolar proteins, α-mannosidase and aminopeptidase I (API) use a novel transport mechanism that is independent of the secretory pathway (Yoshihisa and Anraku, 1990; Klionsky et al., 1992). These polypeptides are probably transported by the same protein sorting machinery, although the specifics of API targeting have been more thoroughly scrutinized.

API is synthesized as a cytoplasmic precursor that is posttranslationally targeted to the vacuole (Klionsky et al., 1992). Upon arrival in the vacuole the amino-terminal pro-region is cleaved by protease B to yield the mature-sized polypeptide (Trumbly and Bradley, 1983; Chang and Smith, 1989; Cueva et al., 1989; Klionsky et al., 1992). Vacuolar local-

1. Abbreviations used in this paper: API, aminopeptidase I; CPY, carboxypeptidase Y; cvt, cytoplasm to vacuole; IB, import buffer; SLM, spheroplast labeling medium; SMD, synthetic minimal medium containing glucose; V-ATPase, vacuolar ATPase; vps, vacuolar protein sorting.
ization of API is independent of a number of the sec and vps gene products that are required for correct sorting of proteins traveling through the secretory pathway to the vacuole (Klionsky et al., 1992). Recently, mutants in the cytoplasm to vacuole targeting pathway (cvr) used by API have been identified in our laboratory (Harding et al., 1995). In cvr mutants vacuolar sorting of API is defective while targeting via the secretory pathway is not disrupted, indicating that these two pathways of vacuolar localization use distinct proteinaceous components.

To begin analyzing the biochemistry of the Cvr pathway, we developed an in vitro import assay for API uptake. Our assay uses a permeabilized cell system whereby spheroplasts are radiolabeled in vivo, and then quickly lysed such that the maturation of the newly synthesized protein occurs in vitro. Maturation of API is dependent on ATP hydrolysis, and results in API becoming sequestered away from soluble components. Maturation of API in vitro also requires a functional V-ATPase, as well as gene products that have previously been shown to be involved in API uptake and processing in vivo.

Materials and Methods

Strains and Plasmids

Saccharomyces cerevisiae yeast strains used in this study were: SEY6210 MATa leu2-3,112 ura3-52 his3-A200 trpl-A901 lys2-801 GAL (Robinson et al., 1988); SEY6211 MATa leu2-3,112 ura3-52 his3-A200 trpl-A901 lys2-801 suc2-29 GAL (Robinson et al., 1988); SEY2101 Drep4 MATa leu2-3,112 ura3-52 ade2-1 suc2-29 Drep4 LEU2 (Bankaitis et al., 1986); THY144 86, derivative of SEY6211 (Harding et al., 1995); vps9 derivative of SEY6210 (Robinson et al., 1988); KMY1094, SEY2101 vac1-4: LEU2 (Morano and Klionsky, 1994). The plasmids pRN1 (centromeric API) and CYJ306-50 have been previously described (Johnson et al., 1987; Klionsky et al., 1992).

Reagents

Oxalylcise was obtained from Zenzogenetics (Corvallis, OR), Expres35S 35S was from Du Pont-NEN Research Products (Boston, MA), Sulfo-NHS-biotin and Avidin agaroase were from Pierce (Rockford, IL), and all other reagents were from Sigma Chemical Co. (Saint Louis, MO). Antisem against API was prepared as described (Klionsky et al., 1992).

Spheroplasting, Cell Labeling, and Immunoprecipitation

Cells were grown to an OD600 of 1.0 in synthetic minimal medium (SM; 0.067% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed). Preparation of spheroplasts was performed as described (Klionsky et al., 1992) except that dithiothreitol treatment was for 20 min and the spheroplasting reactions were performed in spheroplast labeling medium (SLM; 1 M sorbitol, 1% glucose, 1% proline, Wickerham’s salts, pH 7.5 [Guthrie and Fink, 1991], and auxotrophic amino acids and vitamins as needed). After spheroplasting, the spheroplasts were regenerated by incubation at 10 OD/ml in SLM, pH 5.0, for 5 min at 30°C. For labeling, the spheroplasts were pelleted and resuspended in fresh SLM, pH 5.0, at 20 OD/ml and labeled with 4 μCi Expres35S label/OD at 30°C for 5 min. Chase reactions were performed at 30°C for the times indicated by diluting the spheroplasts to 2 OD/ml in SLM containing 2 mM cysteine, 4 mM methionine and supplemented with 0.2% yeast extract. Whole cell labeling and immunoprecipitation reactions were as described previously (Klionsky et al., 1992).

Cell Permeabilization and Protein Import Assays

Spheroplasts were prepared and labeled as above for 5 min, and the chase was initiated by the addition of cold cysteine and methionine. The labeled spheroplasts were immediately divided into 20-OD aliquots and span at 3,000 g for 1.5 min. The media was discarded (see Fig. 1 B), and the spheroplast pellet was permeabilized by resuspending in 1 ml import buffer (IB; 20 mM K-Pipes, pH 6.8, 100 mM sorbitol, 100 mM KCl, 50 mM KOAc, 5 mM Mg(OAc)2; modified from Haas et al., 1994). An ATP regenerating system (1 mM ATP, 40 mM creatine phosphate, and 0.2 mg/ml creatine kinase; Baker and Schekman, 1989) or inhibitors were then added. Time zero samples were taken at this time to show the starting material available for the beginning of the import reaction (data not shown). The permeabilized cells were incubated at 30°C for 2 h, unless otherwise indicated (T fraction).

The import reactions were terminated by pelleting the vacuoles at 5,000 g for 5 min. The supernatant (S) containing cytosol was removed, and the pellet (P) was resuspended in 100 μl 15% Ficoll then overlaid with 1 ml 13% Ficoll and 300 μl 2% Ficoll. All Ficoll solutions were made in IB. The resulting step gradient was spun in a microcentrifuge at 12,000 g for 5 min. A vacuole-enriched float fraction (F) was collected from the 13/2% interface. This fraction was precipitated with 10% trichloroacetic acid and prepared for immunoprecipitation as previously described (Klionsky et al., 1992).

Biotinylation and Avidin Precipitation

To perform biotinylation, the float fractions from six import reactions were pooled and then aliquoted into three tubes. Sulfo-NHS-biotin was dissolved at 1 mg/ml in IB containing 40 mM K-Pipes, pH 7.8. The first sample received buffer only (see Fig. 5, -bio), the second Sulfo-NHS-biotin (+bio) and the third Sulfo-NHS-biotin (final concentration 0.28 mg/ml) and 0.2% Triton X-100 (+bio + TX100). The biotinylation reactions were incubated at room temperature for 30 min. The reactions were quenched by the addition of Tritc base to 100 mM followed by trichloroacetic acid precipitation. Each reaction was then split into two aliquots. The first aliquot was subjected to three successive immunoprecipitation reactions with anti-API antiserum. The second aliquot was immunoprecipitated with anti-API antiserum, then precipitated with avidin agarose, and finally reimmunoprecipitated with anti-API antiserum. Avidin precipitations were incubated for 2 h at 4°C in immunoprecipitation buffer with 30 μl avidin agarose. The avidin beads were washed once in immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA) and eluted with 50 μl elution buffer (20 mM NaPi, pH 7.5, 1 M urea, 2% SDS) for 15 min at 95°C.

Quantitation Methods

A Fuji Fujix BAS 100 Bioimaging analyzer (Fuji Medical Systems, USA; Stamford, CT) was used for quantitation. In Figs. 3 and 4, the amount of API maturation in vitro is reported as the percentage of mature-sized API in each lane (corrected to compensate for the two out of a total of six methionines that are lost when the pro-region is removed). In Figs. 7 and 9 this number has been normalized to the value obtained in a control reaction. This method of comparison is the most consistent because potential differential sample recovery in the fractionation steps and immunoprecipitation reactions would not be reflected in the outcome. Additionally, in each experiment a sample of the total labeled spheroplasts was collected after the 5-min pulse and immunoprecipitated. This sample was used to quantitate the total amount of API precursor present in each import reaction. For calculations of the total amount of API matured, the amount of mature in the float fraction was compared to the total amount of API precursor present in the reaction. This calculation requires a measurement of the yield recovered in the fractionation procedure. From Fig. 1A, after the osmotic lysis 73% of the vacuoles were still intact. Of these, 60% were recovered in the float fraction (equivalent to 44% of the starting material). Therefore, ~59% of the mature API formed by the import reaction is expected to be recovered in this fraction. Successful fractionation experiments consistently have similar yields to those in the experiment shown in Fig. 1 A.

Miscellaneous Methods

Enzyme assays for α-glucosidase and invertase were performed as described (Halvorson and Ellias, 1958; Goldstein and Lampen, 1975). Protease treatments were performed on 2 OD600 equivalents of permeabilized spheroplasts or cell fractions with 50 μg/ml proteinase K in a final volume of 100 μl. Digestions were for 30 min on ice and were stopped by trichloroacetic acid precipitation. SDS-PAGE and fluorography were as described previously (Harding et al., 1995).
Results

**Development of an In Vitro Assay for API Uptake**

To facilitate investigation of the mechanisms used for cytoplasm to vacuole targeting, we decided to reconstitute the vacuolar localization of API in vitro. Because this mode of transport had not been characterized, we designed an in vitro import assay that maintained as much similarity to in vivo conditions as possible. To accomplish this, we pulse-labeled spheroplasts, subjected them to a differential osmotic lysis that lysed the plasma membrane while leaving the vacuole intact, and then followed the maturation of API. Advantages of this approach are that API precursor is synthesized in vivo, so it is likely to attain an import competent conformation, and that all of the original cellular components are still present during the in vitro reaction.

Spheroplasts were prepared from the yeast strain SEY6210 harboring a centromeric plasmid containing the *APE1* gene encoding API. The spheroplasts were pulse labeled with [³⁵S]methionine for 5 min. Cold cysteine and methionine were added, and the cells were immediately pelleted and resuspended in IB, a buffer containing 100 mM sorbitol and a physiological concentration of salts (see Materials and Methods). This treatment causes plasma membrane lysis, while maintaining the integrity of the vacuolar membrane (Fig. 1 A). Since the half-time for API maturation in whole cells is ~45 min (Klionsky et al., 1992), only the precursor form of API is present at the time of lysis (data not shown). To initiate the in vitro reaction, ATP and an ATP regeneration system were added, and the permeabilized cells were incubated at 30°C. After 2 h, the cells were fractionated to enrich for the vacuolar fraction. Briefly, the permeabilized cells were pelleted and resuspended in IB containing 15% Ficoll, overlaid first with 13% Ficoll in IB, and then with 2% Ficoll in IB. The resulting step gradient was subjected to a 5-min spin at 12,000 g. The vacuole-enriched fraction was collected from the 13/2% interface, acid precipitated, resuspended and immunoprecipitated with antisera against API (Fig. 1 B). The resulting proteins were resolved by SDS-PAGE and detected by fluorography.

In whole cells, API is completely matured at a two hour chase point (Fig. 2 A; Klionsky et al., 1992). In contrast, precursor API is incompletely matured in spheroplasts due to the long half-time of import (compare whole cells to spheroplasts in Fig. 2 A). When chased in SLM, we routinely achieve 50% maturation in spheroplasts after a 2-h chase. Because our in vitro system initiates with spheroplasts, we expect a theoretical maximum 50% maturation of the precursor protein. When pulse-labeled spheroplasts are osmotically lysed and subjected to a nonradioactive chase for 2 h in the presence of an ATP regenerating system, mature API is recovered in the vacuole-enriched float fraction (Fig. 2 B). In Fig. 2 B, 10% of the total API synthesized was matured in the in vitro reaction (see Materials and Methods for calculation). Given the 50% theoretical maximum that we anticipate, this level of import is in the range of most published in vitro assays (Baker et al., 1988; Vida et al., 1990; Brodsky et al., 1993).

The amount of cell material used in the flotation step was eight times as much as is shown in the other fractions.

**Figure 1.** Cell permeabilization procedure and in vitro assay design. (A) Spheroplasts were subjected to differential osmotic lysis, followed by fractionation. Supernatant, pellet, and float fractions were collected. The fractionated cells contained a plasmid encoding a carboxypeptidase Y-invertase fusion protein that is targeted to the vacuole (Johnson et al., 1987). Vacuolar recovery was assayed by comparing invertase activity in the fractions. The relative amount of cytosol present in each fraction was assessed by measuring α-glucosidase activity. The data shown are from three replicate permeabilization/fractionation experiments. (B) The scheme represents the protocol used to reconstitute cytoplasm to vacuole transport. IB: import buffer; 100 mM sorbitol, 100 mM KCl, 50 mM KOAc, 5 mM Mg(OAc)₂, 20 mM K-PIPES, pH 6.8. T, total fraction; S, supernatant; P, membrane pellet; F, vacuole-enriched float.

Taking into account the yield of the float procedure, (see Fig. 1 A) the relative load of the float fraction is approximately five times more than the other fractions shown (Fig. 2 B). The mature API copurifies with vacuolar markers in the float fraction (Figs. 1 A and 2 B), and is not detected in the supernatant fraction, even if more concentrated supernatants or longer exposures are examined (mature API is easily seen in both the total and the pellet...
Maturation of API Is Protected in a Membrane-bound Compartment

To demonstrate that we have reconstituted API transport across the vacuolar membrane, it was necessary to show that the mature API produced by this reaction is inside a membrane-bound compartment. Because API is a vacuole-resident protease (and therefore resides in an environment rich in protease activity), it is difficult to digest it completely with exogenously added proteases (Harding et al., 1995). This inherent protease resistance makes a protease-protection assay problematic. Instead, we decided to test for the accessibility of mature API to modification by a water-soluble biotinylated cross-linker, Sulfo-NHS-biotin. If mature API is not protected by a membrane, it would become biotinylated and could be purified by avidinagarose beads. If it is protected by a membrane, it would only be biotinylated in the presence of detergent.

In the absence of detergent, the precursor form of API is precipitated by avidin agarose (Fig. 5 A, +bio). The intermediate-sized API band also precipitates in agreement with its presumed cytosolic location. Mature API was not detected under these conditions. When the membrane integrity was destroyed by the addition of detergent, biotinylated mature API was recovered with the avidin agarose,

Figure 3. Time course of in vitro maturation. Protein import assays and Ficoll step gradients were performed as in Fig. 2 B except that the cells were fractionated at the times indicated in the graph. The percent mature API was calculated by quantitation using a Fuji Phosphorimager and represents the ratio of mature API to total API recovered in the float fraction. Square symbols (□) are maturation in the presence of ATP and the ATP regenerating system, and circles (○) are maturation in the presence of 1 mM ATPγS.

Maturation of API Is Time and Temperature Dependent

The kinetics of the reconstituted in vitro import reaction were examined to determine if they were characteristic of an enzymatic reaction. The standard in vitro import reaction was initiated in the presence of ATP or ATPγS, and incubated for 0 to 4 h. At each time point, the vacuole-enriched float fraction was recovered and analyzed to determine the percentage of mature API. In the presence of ATP, the reaction had a half-time for maturation of 85 min (Fig. 3). Maximum import was seen at 3 h. To ensure that our import reactions were in the linear range, we stopped the reaction at the 2-h time point in all other experiments. The absence of maturation at the early time points indicates that processing of precursor API is not occurring during the fractionation procedure. When the same reactions were performed in the presence of ATPγS only minimal levels of mature API were recovered in the float fraction (Fig. 3). One characteristic of protein transport reactions is that they usually take place at physiological temperatures. We performed a temperature profile on the reconstituted reaction to see if the cytoplasm to vacuole pathway has a similar temperature optimum. As predicted, we found that at both low and high temperatures maturation was inhibited (Fig. 4). Near physiological temperatures, import of API was optimal.
Maturation requires physiological temperature. Import assays were performed as in Fig. 2 B, except that they were incubated for 2 h at the temperature indicated in the graph. Quantitation of the percent mature API in the float fraction was calculated as in Fig. 3.

along with the precursor and intermediate forms (Fig. 5 A, +bio +TX100). Control reactions either lacking NHS-biotin (Fig. 5 A, −bio), or which were quenched with 100 mM Tris base before the addition of NHS-biotin (data not shown) showed no API recovery. Because mature API is not accessible to the biotinylated cross-linker unless the vacuolar membrane is permeabilized by Triton X-100, it must be protected in a membrane-bound compartment.

**Characterization of the API Precursor**

Having established that the mature API formed in our assay was localized in a membrane-bound compartment, we wanted to characterize the population of precursor that is competent for import in our assay. To accomplish this, we separated both labeled and unlabeled permeabilized cells into 5,000 g pellet and supernatant fractions before using them in an import assay. We found that if a labeled pellet fraction was resuspended in IB plus ATP and ATP regenerating system, in the absence of the supernatant fraction, formation of the mature-sized API was only slightly decreased compared to the control reaction where the supernatant was still present (data not shown). These results suggest that the import competent API precursor is already associated with a membrane before the instigation of the import reaction.

To demonstrate that the import competent material was not already inside a membrane-bound compartment, we performed a protease-protection experiment. While the mature API is not easily protease digestible, the pro-region is susceptible to degradation by added proteases. Spheroplasts were labeled and lysed as in a typical in vitro experiment. At time zero of an import reaction, lysed spheroplasts were separated into supernatant and pellet fractions and subjected to protease treatment. In all three fractions the precursor form of API was digested (Fig. 5 B), indicating that it is not located in a membrane-bound compartment. Interestingly, while the precursor in the total and supernatant fractions was only partially degraded, the import competent precursor in the pellet fraction was completely digested suggesting that it has undergone a conformational change. In all cases the relative sensitivity of API to degradation suggests that the protein had not attained its resistant form under the pulse conditions used in this experiment.

**In Vitro Maturation of API in Mutant Yeast Strains**

To confirm the fidelity of our reconstituted system, we examined the maturation of API in vitro in previously characterized yeast mutant strains. The API pro-region is normally cleaved by proteinase B (Klionsky et al., 1992), an enzyme that requires proteinase A for its own activity (Hirsch et al., 1992). To demonstrate that the maturation observed in our in vitro assay was dependent on these vacuolar proteases, we examined API import in a yeast strain in which the PEP4 gene encoding proteinase A had been deleted. As expected, in vitro maturation of API was not observed in this strain (Fig. 6).

Recently our lab has isolated a series of mutants designated cvt that are specifically defective in cytoplasm to vacuole targeting and accumulate precursor API (Harding et al., 1995). We used representative cvt mutants cvt5 and cvt6 for in vitro import assays. In agreement with the in vivo data, maturation of API was not observed in either mutant strain (Fig. 6). In addition to the cvt strains, other yeast mutants affect vacuolar protein delivery. The vps mutants were isolated based on missorting of carboxypep-
Maturation of API Requires a Functional Vacuolar ATPase

Cytoplasm to Vacuole Transport Requires ATP

Many stages of protein transport and membrane translocation have been shown to require energy in the form of ATP (Wickner, 1994). Accordingly, we next investigated the energetics of cytoplasm to vacuole targeting in our permeabilized cell system. As shown in Figs. 2 and 3, API is matured in the presence of ATP, and maturation is inhibited in the presence of ATPγS (Fig. 7, lanes 1 and 2). To confirm the observed requirement for ATP, we depleted ATP from the permeabilized cells by adding either apyrase or the combination of glucose and hexokinase to the import reaction. In both cases, maturation of API was significantly inhibited (Fig. 7, lanes 3 and 4). These results confirm that in vitro assay accurately portrays the in vivo reaction.

Maturation of API Requires a Functional Vacuolar ATPase

Previous results from our laboratory suggested that API localization is inhibited when the V-ATPase is impaired (K. Morano and D. Klionsky, unpublished observation). We examined the maturation of API in vitro in a strain in which a gene encoding one of the peripheral subunits of the V-ATPase had been deleted. The VMA4 gene encodes a 27-kD subunit of the V-ATPase and is required for activity (Nelson and Nelson, 1990; Ho et al., 1993). We prepared spheroplasts from wild-type and Δvma4 mutant strains of yeast, and analyzed them under both in vivo and in vitro import conditions. Maturation of API was greatly reduced both in vivo and in vitro (Fig. 8 A).

Because the Δvma4 mutation results in chronic deacidification, we could not assess whether the observed inhibition of API targeting was a secondary effect or resulted directly from inactivation of the V-ATPase. To circumvent this problem, we used the specific V-ATPase inhibitor bafilomycin A1 (Bowman et al., 1988). Spheroplasts were incubated with 10 μM bafilomycin A1 for 10 min before labeling. This preincubation allows bafilomycin A1 to collapse the vacuolar ΔpH before the spheroplasts are labeled and transport of API begins. In vivo, the effect on API maturation was similar to the results observed with the Δvma4 strain (Fig. 8 B), suggesting that the V-ATPase is directly involved in API import. When spheroplasts labeled in the presence of bafilomycin A1 were used for an in vitro reaction, the in vitro import process was also blocked (Fig. 8 B, in vitro label). In contrast, when bafilomycin A1 was added to an in vitro reaction at the time of cell permeabilization API maturation was not significantly affected (Fig. 8 B, in vitro chase). These results suggest two possibilities: (a) the in vitro reaction that we have reconstituted has already passed the bafilomycin A1-sensitive step before the cell permeabilization step takes place; or (b) the inhibitory ef-
**Discussion**

In vitro reconstitution of biological events is a powerful tool for unraveling the complex biochemistry of cellular processes. With this in mind, we designed an in vitro assay to reconstitute the transport of the vacuolar protein API. We have used the maturation of API by the vacuolar protease B as a marker for correct vacuolar delivery, and found that API maturation in our system is dependent on time (Fig. 3), temperature (Fig. 4), and energy sources (Figs. 7–9). Further, the mature-sized polypeptide colocalizes with a fraction enriched for vacuoles (Figs. 1A and 2B), and is protected in a membrane-bound compartment (Fig. 5A). It is not detected in the soluble fraction (Fig. 2B).

Maturation of API in our system must be occurring in vitro because in vivo maturation is not detected in spheroplasts resuspended in import buffer that has been modified to maintain spheroplast integrity, and it is not inhibited by the addition of ATPγS (Fig. 2A). Further, we found that the reconstituted system uses the same proteinaceous components as in vivo sorting because mutant yeast strains that can not mature API in vivo also do not mature API in vitro (Figs. 6 and 8).

The starting material for the in vitro reaction was synthesized in vivo by pulse labeling intact spheroplasts for 5 min, followed by osmotic lysis of the plasma membrane. We expect that the newly synthesized precursor embarks on its normal import pathway, and at the time of cell lysis, has moved through the first steps of the pathway. We found that the API precursor that was competent for maturation in our system was already found in the membrane pellet when we began the in vitro reaction (data not shown). Additional cytosolic components beyond those that may be associated with the pellet fraction were not required for the in vitro maturation event. These data suggest that we have reconstituted a post-membrane binding transport event. Because precursor API is accessible to exogenously added protease at the beginning of the in vitro reaction (Fig. 5B), we believe we have reconstituted the membrane translocation step of the import process.

Biochemical reconstitution of protein sorting reactions has been the subject of extensive research. Data from these investigations is beginning to provide considerable information regarding the functions of specific polypeptides that direct these cellular reactions. Typical in vitro assays achieve yields in the range of 10–50% of the available polypeptide (Baker et al., 1988; Ruohola et al., 1988; Vida, et al., 1990; Brodsky et al., 1993). For this reason, in vitro assays reconstituting vacuolar localization through the secretory pathway present a particular challenge because of the numerous steps involved. Many individual steps along this pathway including translocation into the ER (Brodsky et al., 1993; Panzner et al., 1995), ER to Golgi (Beckers et al., 1987; Baker et al., 1988; Ruohola et al., 1988), intra-Golgi (Balch et al., 1984; Rothman, 1987) and ER to Golgi. Maturation of API in our system must be occurring in vitro because in vivo maturation is not detected in spheroplasts resuspended in import buffer that has been modified to maintain spheroplast integrity, and it is not inhibited by the addition of ATPγS (Fig. 2A). Further, we found that the reconstituted system uses the same proteinaceous components as in vivo sorting because mutant yeast strains that can not mature API in vivo also do not mature API in vitro (Figs. 6 and 8).

The function of the vacuolar ATPase was found to be essential for correct API targeting to occur both in vivo and in vitro. In either a yeast strain where the gene encoding one of the peripheral subunits of the V-ATPase had been deleted (Δvma4), or when the V-ATPase was chemically inactivated by the inhibitor bafilomycin A₁, maturation was impaired (Fig. 8). Since inhibition of the import reaction occurred after only a 10-min incubation with bafilomycin A₁, an energized vacuolar membrane is probably a direct requirement for this transport reaction to take place. There are several possible models to explain the contribu-

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**Table 1**

| GTP (10 mM) | - | - | + |
| GDP (10 mM) | + | - | + |
| GTPγS (0.1 mM) | + | - | + |

**Relative % Mature:**

| 12 | 100 | 100 | 100 |

---

**Figure 9**

API transport involves a GTP-binding protein. Import experiments were performed as in Fig. 6. All reactions contained 1 mM ATP and an ATP regenerating system. The reactions were supplemented with GTP, GDP, and GTPγS as indicated. The percent mature API in each float fraction was calculated as in Fig. 3, and compared to the percent mature in a control reaction (data not shown) and is shown below each corresponding lane. The positions of precursor and mature API are indicated.
tion of the ΔpH in this reaction. The ΔpH could be required for productive association of precursor API with the vacuolar membrane, or for the association/assembly of a component of the transport machinery. Alternatively, it could act as an energetic component of the reaction. Interestingly, bafilomycin A₁ only affected the in vitro maturation reaction if it was added before the cells were labeled. Possibly, the in vitro reaction that we have reconstituted has already passed the bafilomycin A₁-sensitive step. The use of bafilomycin A₁ and additional inhibitors may help us to order the steps involved in the cytoplasm to vacuole protein import pathway.

The temperature profile of API transport (Fig. 4) may also provide insight into the mechanism involved in API import. Protein transport reactions involving vesicle fusion are usually more sensitive to low temperatures than transport reactions where the translocating polypeptide crosses the lipid bilayer directly. For example, protein import into the ER proceeds at 10°C, while transport to the Golgi is severely impaired (Baker et al., 1988), and autophagic protein uptake requires temperatures of at least 20°C in mammalian cells (Seglen and Bohley, 1992). The fact that significant API maturation did not take place at 14°C is suggestive of a vesicle-mediated transport event. The role and origin of putative vesicular intermediates that are involved in API import are under investigation.

GTP-binding proteins are known to be involved in many vesicular-mediated protein delivery events (Rothman, 1994). We found that both GTPγS and GDPβS act as inhibitors of the Cvt pathway (Fig 9). Inhibition of import by both GTPγS and GDPβS could be reversed by excess GTP but not by excess ATP, suggesting that GTP is specifically involved in this reaction. Neither GTP nor GDP had any effect on the import reaction themselves. This is probably because the yeast cytosol present in the reaction contains a sufficient level of GTP for transport to proceed. In addition, the presence of an ATP regenerating system allows GDP to be phosphorylated to GTP by diphosphonucleotide kinase. The fact that the reaction is inhibited by both a GTP and a GDP analogue suggests that GTP-binding protein cycling is required. One possible model is that the GTP-bound form is necessary for recruitment of an essential component of the translocation machinery, and that this component must then be released from the GTP-binding protein via GTP hydrolysis for the reaction to proceed further (Bourne, 1988).

The yeast vacuole is analogous to the mammalian lysosome. Both organelles are involved in protein turnover, and appear to employ similar mechanisms of acquiring proteins. Nonselective autophagy has been shown to direct soluble polypeptides to both the mammalian lysosome (Seglen and Bohley, 1992) and the yeast vacuole (Baba et al., 1994). Yeast autophagy mutants have been identified, but this process has not been reconstituted in vitro (Tsukada and Ohsumi, 1993; Thumm et al., 1994). Selective uptake of proteins destined for degradation has been reconstituted in lysosomes purified from mammalian cells (Terlecky and Dice, 1993). The extent to which the cytoplasm to vacuole protein targeting pathway that we are investigating overlaps with these processes, is not known. Once the degree of overlap between the Cvt pathway and these processes has been determined, investigations utilizing our in vitro assay, coupled with analysis of the cvt mutants will help elucidate the mechanism employed by both cytoplasm to vacuole targeting and related pathways.

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