In Vitro Reconstitution of Intercompartmental Protein Transport to the Yeast Vacuole

Thomas A. Vida, Todd R. Graham, and Scott D. Emr

Division of Biology 147-75, California Institute of Technology, Pasadena, California 91125

Abstract. Toward a detailed understanding of protein sorting in the late secretory pathway, we have reconstituted intercompartmental transfer and proteolytic maturation of a yeast vacuolar protease, carboxypeptidase Y (CPY). This in vitro reconstitution uses permeabilized yeast spheroplasts that are first radiolabeled in vivo under conditions that kinetically trap ER and Golgi apparatus-modified precursor forms of CPY (p1 and p2, respectively). After incubation at 25°C, up to 45% of the p2CPY that is retained in the perforated cells can be proteolytically converted to mature CPY (mCPY). This maturation is specific for p2CPY, requires exogenously added ATP, an ATP regeneration system, and is stimulated by cytosolic protein extracts. The p2CPY processing shows a 5-min lag period and is then linear for 15–60 min, with a sharp temperature optimum of 25–30°C. After hypotonic extraction, the compartments that contain p2 and mCPY show different osmotic stability characteristics as p2 and mCPY can be separated with centrifugation into a pellet and supernatant, respectively. Like CPY maturation in vivo, the observed in vitro reaction is dependent on the *PEP4* gene product, proteinase A, which is the principle processing enzyme. After incubation with ATP and cytosol, mCPY was recovered in a vacuole-enriched fraction from perforated spheroplasts using Ficoll step-gradient centrifugation. The p2CPY precursor was not recovered in this fraction indicating that intercompartmental transport to the vacuole takes place. In addition, intracompartmental processing of p2CPY with autoactivated, prevacuolar zymogen pools of proteinase A cannot account for this reconstitution. Stimulation of in vitro processing with energy and cytosol took place efficiently when the expression of *PEP4*, under control of the *GAL1* promoter, was induced then completely repressed before radiolabeling spheroplasts. Finally, reconstitution of p2CPY maturation was not possible with *vps* mutant perforated cells suggesting that *VPS* gene product function is necessary for intercompartmental transport to the vacuole in vitro.

VESICLE-MEDIATED protein transport through the secretory pathway is an essential process in eukaryotic cells. Nascent proteins initiate the secretory pathway from the cytoplasm as translation exposes the proper signal sequence for translocation across the ER membrane (Walter et al., 1984). Proteins that leave the ER move to the Golgi complex encountering at least three distinct regions, the *cis*-, medial-, and *trans*-Golgi cisternae (Pfeffer and Rothman, 1987). As proteins transit through the Golgi complex, they not only receive a variety of post-translational modifications, but also are sorted and then routed via vesicle carrier to their correct intracellular and extracellular destinations (Farquhar, 1985; Griffiths and Simons, 1986). Considerable effort has been devoted toward understanding the mechanisms and cellular machinery that direct these protein sorting and delivery events.

The yeast lysosome-like vacuole receives its content of hydrolytic enzymes via a transport pathway similar to that found in mammalian cells (Klionsky et al., 1990). Studies with the temperature-sensitive *sec* mutants and mutants defective in processing and maturation of vacuolar zymogen precursors have defined the organelles involved in compartmental delivery of these proteins (Jones, 1977; Schekman, 1985; Ammerer et al., 1986; Woolford et al., 1986). Carboxypeptidase Y (CPY)[1] serves as the model vacuolar protein in most of these studies. Like all proteins entering the secretory pathway, CPY begins its passage to the vacuole after translocation from the cytoplasm into the ER lumen. The first CPY precursor, p1CPY, initially resides in the ER since it only has core-glycosylated oligosaccharide side chains (Stevens et al., 1982; Trimble et al., 1983); its appearance is blocked in mutants defective in ER translocation (Deshaies and Schekman, 1987; Toyn et al., 1989); and p1 accumulates at the nonpermissive temperature in *sec* mutants that are defective in protein transport from the ER (Stevens et al., 1982). Formation of the second CPY precursor, p2CPY, requires transport from the ER to the Golgi complex where the

1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; WIMP, Wickerham's minimal proline; YE, yeast extract.

plCPY precursor acquires 2 kD of mass with specific addition of mannose residues to the core-oligosaccharides. The p2CPY precursor does not form at the nonpermissive temperature in *sec* mutants defective in protein transport out of the ER, but will form when the block is relieved by shifting cells to the permissive temperature (Stevens et al., 1982). Furthermore, the mannosyltransferases responsible for this addition are localized in the Golgi complex (Esmon et al., 1981; Cunningham and Wickner, 1989; Abeijon et al., 1989). The final mature form of CPY (mCPY) is produced from the p2 precursor by proteolytic removal of an 8-kD propeptide located at the amino terminus of the protein (Hasilik and Tanner, 1978). Present data are most consistent with this final posttranslational processing event occurring concomitant with p2CPY's arrival in the vacuole lumen. Only mCPY and not pl or p2CPY are found in purified vacuoles from wild-type cells; mCPY does not appear in *sec* mutants defective in transport from the ER or Golgi complex at the nonpermissive temperature (Stevens et al., 1982; Franzusoff and Schekman, 1989); and the processing enzymes responsible for p2CPY maturation, proteinases A and B, are located in the vacuole lumen where together they catalyze efficient propeptide cleavage (Mechler et al., 1987). Propeptide processing, however, is not required for vacuole delivery of CPY as purified vacuoles from *pep4* (the structural gene for proteinase A) mutant yeast strains contain the p2 precursor (Stevens et al., 1982).

Vacuolar proteins in yeast, like lysosomal proteins in mammalian cells, transit through a common set of early compartments shared with secretory proteins (Stevens et al., 1982; Johnson et al., 1987). However, in the Golgi complex vacuolar/lysosomal proteins are sorted away from proteins destined for the cell surface. The genetic basis for *v*acuole *p*rotein *s*orting in yeast has been addressed by isolating *vps* mutants (Bankaitis et al., 1986; Rothman and Stevens, 1986; Robinson et al., 1988; Rothman et al., 1989). These fall into >40 complementation groups whose principle phenotype is the secretion of vacuolar precursor proteins after they have passed through the Golgi complex. Thus, many of the *VPS* gene products are implicated in routing proteins like CPY from the Golgi complex to the vacuole. The large number of *vps* complementation groups suggest that the protein transport pathway between the Golgi complex and the vacuole is a complex multistep process.

Detailed biochemical study of the many events associated with organelle-to-organelle protein transport is possible through reconstituted assays. Reconstitution of vesicle-mediated protein transport between Golgi cisternae in vitro using a cell-free system has resulted in a detailed understanding for this event (Balch et al., 1984; Weidman et al., 1989; Pfanner et al., 1990). Additionally, the recent development of procedures that permeabilize the plasma membrane of cells to macromolecules has permitted the study of other complex interorganellar protein transport processes in vitro (Beckers et al., 1987; Simons and Virta, 1987). These procedures create "semi-intact," or perforated cells from which soluble cytoplasmic components can be removed while intact, functional organelles are retained. Perforated cell systems can allow for reconstitution of individual interorganellar transport steps, making the complexity of the process in vivo amenable to more direct biochemical analysis. Indeed, the role of cytoplasmic factors and certain *SEC* gene products are now being understood at the biochemical level with the use of reconstituted in vitro assays designed to measure their activities (Baker et al., 1988; Ruohola et al., 1988; Goud et al., 1988; Hicke and Schekman, 1989; Clary et al., 1990).

Virtually nothing is understood at the biochemical level about the steps that take place in vivo as vacuolar precursor proteins move from the late secretory pathway to the vacuole. Here, we describe the in vitro reconstitution of vacuolar protein transport using perforated yeast cells. This process requires ATP hydrolysis, cytosolic protein factor(s), organelle integrity, and involves intercompartmental transfer from a donor compartment to an acceptor compartment, the vacuole.

Materials and Methods

Strains, Media, and Plasmid Constructions

The yeast strains used were SEY2101 *MATa ura3-52 leu2-3,112 suc2-Δ9 ade2-1* (Emr et al., 1983); SEY2101.1 *MATa ura3-52 leu2-3,112 suc2-Δ9 ade2-1 Δpep4::LEU2* (Klionsky et al., 1988); DKY6224 *MATa ura3-52 trp1-Δ901 his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 Δpep4::LEU2* (Klionsky et al., 1988); SEY6210 *MATα ura3-52 trp1-Δ901 his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 GAL* (Robinson et al., 1988); SEY6211 *MATa ura3-52 trp1-Δ901 his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 GAL* (Robinson et al., 1988); and JHRY20-2C *MATa leu2-3,112 ura3-52 his3-Δ200 Δprc1::HIS3 Δpep4::LEU2* (Valls et al., 1987). Wickerham's minimal proline (WIMP) media was as described previously (Wickerham, 1946; Johnson et al., 1987) but supplemented with 0.2% yeast extract (YE). Rich media was either 2% bactopeptone, 1% yeast extract, and 2% glucose (1× YPD), 2× YPD, or 1× YP with 5% glucose.

To construct a single copy plasmid with *PEP4* under control of the galactose-inducible promoter (*GAL1*) pSEYC68-GAL was used, which contains the *GAL1-GAL10* promoter region from pBM150 (Johnston and Davis, 1984) cloned into the Eco RI–Bam HI sites of pSEYC68 (Herman, P. K., and S. D. Emr, manuscript submitted for publication). The Bam HI-Sal I fragment from pSEYC306-*PEP4* (Klionsky et al., 1988) was subcloned into M13mpl9. Oligonucleotide site-directed mutagenesis (Kunkel et al., 1987) was used to place a second Bam HI site between nucleotide positions -9 and -4 relative to the initiating codon in *PEP4*. The resulting 3.0-kb Bam HI–Sal I fragment was then subcloned into pSEYC68-GAL to give p*GAL1-PEP4*. This was transformed into DKY6224, which was maintained on selective minimal plates with glucose or galactose as the sole carbon source.

Preparation of Radiolabeled, Perforated Yeast Spheroplasts

Strains were grown to an OD_{600} (1–2 × 10^7 cells/ml) of 1.0–1.2 in WIMPYE media. The standard amount of cells was 100 OD_{600} units. The cells were harvested by centrifugation at 3,000 *g* for 5 min, resuspended to 10 OD_{600} units/ml in 100 mM Tris-HCl pH 9.4, 10 mM DTT and incubated for 10 min at room temperature. After harvesting the cells as above, they were resuspended to 30–50 OD_{600} units/ml in WIMP media containing 1.0 M sorbitol and 20 mM Tris-HCl, pH 7.5. The cell wall was digested with oxalyticase (Enzogenetics, Inc., Corvallis, OR) at 20–40 U/OD of cells for 15–20 min at 30°C. The conversion to spheroplasts was monitored by determining the OD_{600} of a 1:100 dilution into water and was routinely >90%.

The spheroplasts were harvested by centrifugation at 3,000 *g* for 5 min and then resuspended to 20–30 OD_{600} units/ml in WIMP media containing 1.0 M sorbitol. The suspension was agitated at 30°C for 1–5 min, Tran [^{35}S]-label (ICN Radiochemicals, Inc., Irvine, CA) was then added to 250–350 μCi/ml and the cells were pulsed for 4.5 min followed by a chase of 30 s with methionine and cysteine (10 mM each). After the 30-s chase period, the cells were placed immediately in an ice water bath and gently mixed intermittently for 4 min. All subsequent steps were performed ice cold. For some experiments, cells were converted to spheroplasts and labeled in YNB media (0.67% yeast nitrogen base without amino acids, 2% glucose, and 1.0 M sorbitol) because this gave more consistent incorporation of radiolabel in a variety of strains. Similar results were obtained with both WIMP and YNB labeling media.

Aliquots of 25 OD_{600} units of cells were removed into 1.5 ml polypropylene tubes and collected by centrifugation at 13,000 *g* for 20 s. The cells were washed three times by resuspension in 1 ml of 1.0 M sorbitol, containing 1× buffer salts (20 mM Hepes-KOH pH 6.8, 150 mM potassium acetate, and 5 mM magnesium acetate). After the final wash, the cells were resuspended in 0.1 ml of the same buffer and placed ~8 cm above liquid N_2 in a sealed thick-walled styrofoam container for 30–45 min, then stored until use at −70°C.

Cells were removed from the freezer and the frozen cell pellet was thawed at 25°C for 30–45 s. The thawed cells were placed on ice, resuspended to 1 ml in ice-cold transport buffer (0.25 M sorbitol, with 1× buffer salts) and left on ice for 3–5 min. The cell suspension was centrifuged at 13,000 *g* for 15–20 s and the supernatant was removed and saved. The cell pellet was resuspended to 1 ml in 50 mM sorbitol with 1× buffer salts, mixed gently, and incubated on ice for 2–3 min. The cells were collected by centrifugation as before and the supernatant was saved. The cell pellet was washed an additional time in 1 ml of transport buffer, incubated for 3–5 min, recentrifuged, and the supernatant was again saved. The final cell pellet was resuspended to 100 OD_{600} units/ml in transport buffer. The three supernatants were pooled.

For induction/repression of *PEP4* expression, DKY6224 (harboring $p^{GAL1PEP4}$) was grown overnight in WIMPYE with 2% galactose and 0.2% glucose. The cells were then harvested, washed once with sterile distilled water, once with WIMPYE media containing 2% galactose (#G-0750; Sigma Chemical Co., St. Louis, MO), subcultured in the same media, and grown for 12–18 h at 30°C to an OD_{600} of 1.0–2.0. After this induction in galactose, the cells were harvested, washed once with sterile distilled water, once with WIMPYE media containing 2% glucose, resuspended in the same media, and grown for 4 h (two to three generations) at 30°C to an OD_{600} of 1.0–1.2. A parallel subculture was treated similarly but was not washed with water and glucose media, rather it was maintained exclusively in galactose media. Likewise, for complete repression, DKY6224 (harboring $p^{GAL1PEP4}$) was cultured in WIMPYE media that always contained glucose as the carbon source. These three cultures were converted to spheroplasts as described above and radiolabeled with Tran[^{35}S]-label for 7 min, chased with methionine/cysteine (5 mM each) for 6 min at 20°C, and frozen/thawed/washed as described above.

Transport Reactions and Immunoprecipitations

All reactions were prepared on ice. Standard conditions for each reaction contained 5 μl of a 10× ATP regeneration system (10 mM ATP, 400 mM phosphocreatine, and 2 mg/ml phosphocreatine kinase), 150 μg of a cytosolic protein extract (see below), and 1.5–2.0 OD_{600} units of perforated cells in a final volume of 50 μl. The reactions were then placed at 25°C for 60 or 90 min. To stop the reactions, they were placed on ice and SDS and NP-40 (2% final each) plus 1 mM PMSF was added and then they were immediately boiled for 5 min. When using TCA (10% final) to stop, the reaction mixtures were incubated for 5–10 min on ice, subjected to centrifugation at 13,000 *g* for 5–10 min, washed twice with acetone, dried, resuspended in 50 μl of transport buffer plus 20 μl of 5% SDS/NP-40, and boiled for 5 min. After boiling in detergents, 1 ml of IP buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 mM EDTA, and 0.5% Tween-20) was added and the diluted lysate was subjected to centrifugation at 13,000 *g* for 5–10 min. The cleared lysate was then incubated with antisera and 60 μl of a 3.5% (wt/vol) suspension of protein A-Sepharose for 3–16 h with constant agitation at 4°C. The protein A-Sepharose was collected by centrifugation at 13,000 *g* for 1 min and washed with 1 ml of IP buffer followed by a second wash with IP buffer minus 0.5% Tween-20. The washed protein A-Sepharose pellets were boiled in sample buffer and subjected to electrophoresis in a 9% SDS-polyacrylamide gel. The gel was fixed, treated with Autofluor (National Diagnostics, Inc., Somerville, NJ), dried, and autoradiographed. Gels were quantitated using a scanning laser densitometer (model 2202; LKB instruments, Gaithersburg, MD) equipped with integrating software (Gelscan; LKB Instruments).

Preparation of Cytosolic Extracts

The usual strains for making cell-free extracts were either DKY6224 or JHRY20-2C. The cells grown to an OD_{600} of 2–5 in 2× YPD or 1× YP with 5% glucose. The cells (2,000–5,000 OD_{600} units) were harvested and resuspended in 250 ml of 0.1 M Tris-HCl pH 9.4 plus 25 mM β-mercaptoethanol and incubated for 10 min at room temperature. After harvesting, the cells were resuspended to 50 OD_{600} units/ml in 1× YPD, 1.0 M sorbitol, and 20 mM Tris-HCl pH 7.5. Oxalyticase was then added (5–10 U/OD_{600} unit) and the cells were agitated at 30°C for 30 min. The spheroplasts formed in this time were harvested, washed once in 1.0 M sorbitol, and washed once in transport buffer plus 1 mM DTT. One gram of acid-washed glass beads and 0.125 ml of transport buffer containing 1 mM DTT was added for every 250 OD_{600} units of cells. The suspension was then vigorously agitated on a vortex mixer at 1-min intervals for a total of 5–10 min. The lysed cells were centrifuged at 3,000 *g* for 5 min and the supernatant was then centrifuged at 40,000 *g* for 15 min. This 40,000 *g* supernatant was then centrifuged at 100,000 *g* for 30–60 min. The supernatant after 100,000 *g* (S100) was put into 100-μl aliquots, frozen in liquid N_2, and stored at −70°C. Protein concentrations were determined using the Bradford method (1976) and typically were 12–16 mg/ml.

Some extracts were made from large scale perforated cell preparations. After making spheroplasts, 1,000–1,300 OD_{600} units of cells were frozen slowly over liquid N_2 and stored at −70°C as one large cell pellet. After quickly thawing, the cells were washed to remove cytosolic proteins (see above) with 25–30-ml portions of the wash buffers containing 1 mM DTT. The three washes were pooled (~95 ml), solid ammonium sulfate (Bethesda Research Laboratories, Gaithersburg, MD) was added incrementally to 75–80% saturation, and stirred on ice for 20–30 min after the last addition of salt. The precipitate was collected by centrifugation at 15,000 *g* for 20 min, the pellet was rinsed once with 80% saturated ammonium sulfate, and recollected by centrifugation at 15,000 *g* for 5 min. The rinsed pellet was carefully dissolved in 2–3 ml total volume of 250 mM sorbitol, 1× buffer salts, 1 mM DTT, and applied to a 15-ml desalting column (Bio-Gel P-6DG; Bio-Rad Laboratories, Richmond, CA) equilibrated in the same buffer. Fractions of 0.6 ml were collected and the peak of A_{280} material was pooled (4–6 ml), which was immediately clarified with centrifugation at 250,000 *g* for 30 min. The supernatant was collected, put into 100–125-μl aliquots, quick-frozen in liquid N_2, and stored at −70°C. These extracts had protein concentrations of 7–12 mg/ml.

Fractionation of Perforated Cells

Hypotonic Extraction. Spheroplasts were radiolabeled and perforated as described above. Larger scale (350–500 μl) reactions were incubated for 90 min at 25 or 0°C. After incubation, the reactions were chilled on ice for 5 min, layered over a cushion of 1.0 M sorbitol containing 20 mM Hepes-KOH pH 6.8, 150 mM potassium acetate, and 5 mM magnesium acetate, and collected by centrifugation at 13,000 *g* for 5 min at 4°C. The pellets were resuspended in 250 μl of 20 mM sorbitol, 20 mM Hepes-KOH pH 6.8, 75 mM potassium acetate, 2.5 mM magnesium acetate and subjected to vigorous agitation for 1 min; then 750 ml of transport buffer was added. An aliquot of this suspension was removed and the remainder was subjected to sequential centrifugation at 1,000, 13,000, and 100,000 *g*. Initial experiments indicated that critical separation was achieved after centrifugation at 13,000 *g* so the other *g* forces were discontinued. Each fraction was precipitated with TCA and immunoprecipitated as described above.

Ficoll Step Gradient. Perforated cells (prepared as described above) were subjected to Ficoll flotation gradients. Radiolabeled (12.5–25 OD_{600} units) and unlabeled cells (25–50 OD_{600} units) were incubated at 25°C with ATP, an ATP regeneration system, and cytosolic extracts (scaled up proportionately) for 90 min. Incubations at 0°C with ATP, an ATP regeneration system, and cytosolic extracts or at 25°C with transport buffer alone were performed in parallel as controls. After incubation, the entire volume of the reactions was layered on top of 2 vol of 0.8 M sorbitol, 15 mM triethanolamine-acetic acid pH 7.3, 1 mM EDTA, and subjected to centrifugation at 13,000 *g* for 5 min. The pellet was resuspended in 125 μl of 0.4 M sorbitol, 15 mM triethanolamine-acetic acid pH 7.3, 1 mM EDTA, mixed with pipetting up and down 10–25 times, and incubated 10 min at 25°C (Makarow, 1985). The lysate was then subjected to centrifugation at 500 *g* for 30 s and the supernatant was saved. The pellet was resuspended in 125 μl of 0.4 M sorbitol, 15 mM triethanolamine-acetic acid pH 7.3, 1 mM EDTA, incubated 5 min at 25°C, and subjected to centrifugation at 500 *g* for 30 s. The 500-*g* supernatants were pooled (250 μl) and the following solutions were added: 250 μl of 0.4 M sorbitol, 15 mM triethanolamine-acetic acid pH 7.3, 1 mM EDTA; 500 μl of 30% Ficoll (Pharmacia-LKB Biotechnology Inc., Piscataway, NJ) in 0.1 M imidazole-HCl pH 6.5. After mixing gently, 10% of the total lysate was saved (0.1 ml) and the remainder (0.9 ml) was layered on top of 30% Ficoll, 0.1 M imidazole-HCl pH 6.5, 0.2 M sorbitol (0.25 ml) in an Ultra-Clear tube (13 × 51 mm; Beckman Instruments, Fullerton, CA). The following Ficoll solutions were layered on top of the lysate all in 0.1 M imidazole-HCl pH 6.5, 0.2 M sorbitol: 12.5% (0.5 ml), 10% (0.5 ml), 8% (0.5 ml), 6% (1.0 ml), 4% (1.0 ml), and 0% (0.4 ml) for a total volume of 5 ml. The gradients were subjected to

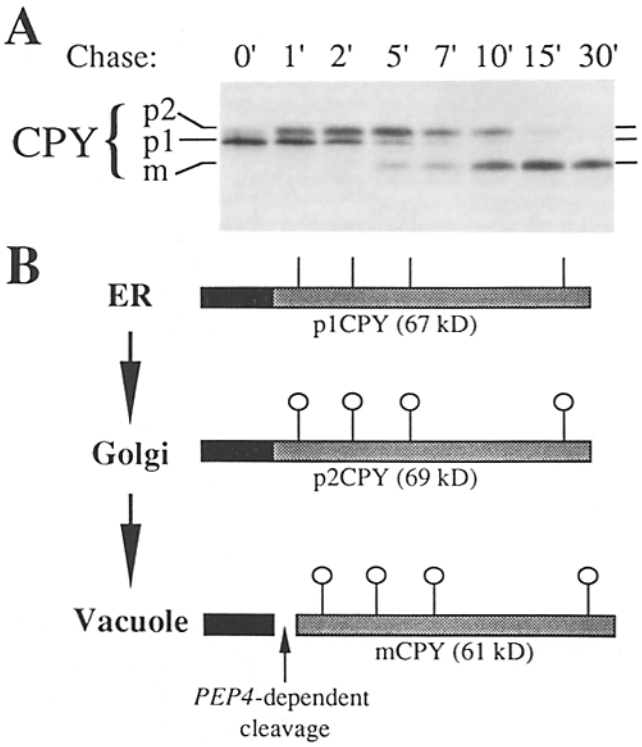

Figure 1. Compartmentalized biosynthesis and processing of CPY in vivo. (*A*) Yeast cells (SEY2101) were grown and converted to spheroplasts as described in Materials and Methods. The spheroplasts were pulse-labeled with Tran[^{35}S]-label for 5 min and chased with unlabeled methionine and cysteine for the indicated times at 30°C. To stop the chase, the spheroplasts were diluted in an excess of ice-cold buffer containing 10 mM each NaN_3 and NaF. Immunoprecipitations for CPY were performed and the immunoprecipitates were subjected to SDS-PAGE as described in Materials and Methods. (*B*) Schematic diagram depicting the compartmentalized modifications that take place on CPY during movement from the ER through the Golgi complex and to the vacuole. The solid bar represents the amino-terminal propeptide (91 amino acids) and the stippled region signifies the mature portion of CPY (421 amino acids). Vertical lines on p1CPY represent the four core oligosaccharides added in the ER, whereas circles designate mannose addition in the Golgi complex.

centrifugation at 75,000 *g* for 2 h at 10°C in a SW 50.1 rotor (Beckman Instruments). 10 0.5-ml fractions were carefully removed from the top of the gradients and placed in 1.5 ml polypropylene tubes. The 10% aliquot of the lysate and all ten radiolabeled fractions were precipitated with TCA (200 μg BSA as carrier), immunoprecipitated with CPY antiserum, subjected to SDS-PAGE, and fluorography as described above. The 10% aliquot of the unlabeled lysate and each gradient fraction were assayed for α-mannosidase (Opheim, 1978), CPY (Stevens et al., 1986), NADPH cytochrome-*c* reductase (Kubota et al., 1977), and guanosine diphosphatase, GDPase (Abeijon et al., 1989).

Microscopy

The general methods have been described previously (Banta et al., 1988; Pringle et al., 1989). Yeast spheroplasts, at 25–30 OD_{600} units/ml were stained at 30°C with 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (dichlorocarboxyfluorescein, 50 μM final; Molecular Probes, Inc., Beaverton, OR) for 15 min in media containing 1.0 M sorbitol and buffered to pH 4.0 with 0.1 M citrate-KOH. The cells were then washed in isotonic buffer, frozen slowly over liquid N_2, and stored at −70°C until use as described above. Cells were stained with rhodamine-dextran (Sigma Chemical Co.) at 1 mg/ml for 5–10 min on ice, harvested, and resuspended in appropriate buffers or media depending on experimental circumstances.

Results

Compartmentalized Biosynthesis and Processing of CPY In Vivo

The posttranslational modifications that occur on CPY during transit through early secretory organelles to the vacuole result in electrophoretic mobility differences. These differences can be seen with pulse-chase radiolabeling analysis and are indicative of CPY's compartmental location. During a 5-min pulse with [^{35}S]methionine, the ER form of CPY, p1, predominated with just a slight amount of poorly resolved p2CPY (Fig. 1 *A*). After just 1 min of chase, p2CPY increased significantly and became clearly resolved from p1CPY, corresponding to addition of mannose residues in the Golgi complex. As the chase proceeded, mCPY appeared, which was easily separated from p2CPY after propeptide cleavage (Fig. 1 *A*, 5–30 min of chase). Even after 5 min of chase, p1 and p2CPY predominated, whereas mCPY was only ~10% of the total, demonstrating that the p2 precursor of CPY represented a significant pool after short chase times at 30°C in wild-type cells (Fig. 1 *A*). With these in vivo kinetics, significant amounts of p1 and p2CPY can be trapped in prevacuolar compartments. These precursor forms could be used as substrates to monitor reconstitution of protein transport to the vacuole using "semi-intact" yeast spheroplasts.

Preparation of Perforated Yeast Spheroplasts

Semi-intact or perforated cells are ideal for reconstituting complex organelle-to-organelle protein transport processes because they can maintain the spatial and structural architecture of intracellular organelles (Beckers et al., 1987; Simons and Virta, 1987; Baker et al., 1988; Ruohola et al., 1988). Yeast cells are amenable to permeabilization techniques after removal of the cell wall. The resulting spheroplasts have been perforated with either slow-freezing and thawing in hypotonic buffer (Baker et al., 1988) or hypotonic washing (Ruohola et al., 1988). These previous methods did not result in quantitative conversion of spheroplasts to a perforated state. Complete conversion was required to remove background maturation of CPY that took place in unbroken cells during subsequent in vitro incubations.

A combination of slow freezing in isotonic buffer, thawing, and sequential washing with hypotonic buffers was empirically determined to be capable of quantitatively rendering spheroplasts devoid of cytosolic protein. The freezing conditions in isotonic buffer were chosen not only for convenience (long-term storage), but also to maintain spheroplasts as intact as possible while frozen. Thawed radiolabeled spheroplasts were washed three times with hypotonic buffers, removing 95% or more of the cytosolic protein markers, glucose-6-phosphate dehydrogenase (G6PDH) and phosphoglycerol kinase (*PGK*, Fig. 2 *A*). This suggested the cells were either selectively depleted of soluble cytoplasmic protein or that they completely lysed during the freezing, thawing, and washing scheme. However, since they were radiolabeled under conditions that kinetically trap p1 and p2CPY, the lumen of the ER and Golgi compartments were marked so the integrity of these organelles was easily assessed. The cells retained 75 and 90% of p1 and p2CPY respectively, suggesting that very little breakage of the ER and Golgi complex membranes had occurred (Fig. 2 *A*). The

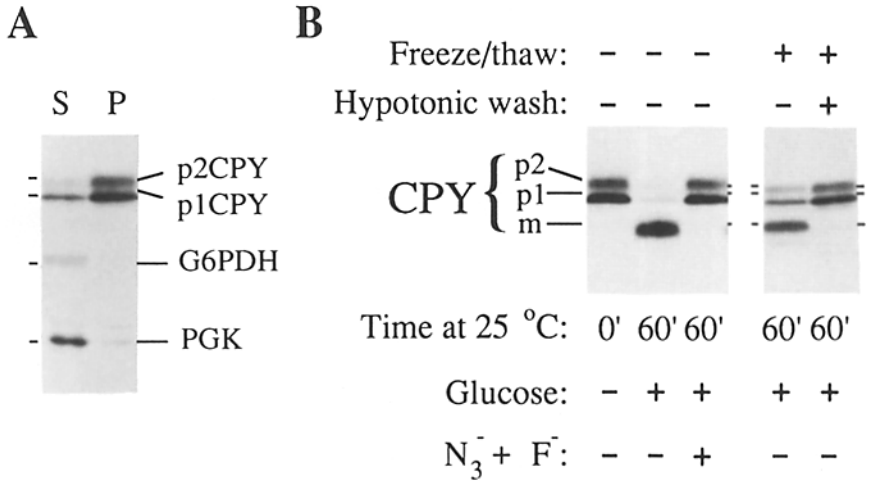

Figure 2. Preparation of perforated spheroplasts. (*A*) Yeast spheroplasts (SEY6211 or SEY6210) were prepared, radiolabeled, frozen and thawed, and washed as described in Materials and Methods. Equivalent amounts of the final triple-washed perforated cell pellet (*P*) and the pooled supernatant washes (*S*) were coimmunoprecipitated with antisera to glucose-6-phosphate dehydrogenase (*G6PDH*), phosphoglycerol kinase (*PGK*), carboxypeptidase Y (*CPY*), analyzed on an SDS-polyacrylamide gel, fluorographed, and quantitated with densitometry. More than 95% of G6PDH and PGK were washed free from the spheroplasts, whereas 90% of p2CPY and 75% of p1CPY were retained in the perforated cell pellet. (*B*) Intact spheroplasts were radiolabeled as described above. The spheroplasts were then incubated with 5% glucose, NaN_3, and NaF (10 mM each), before and after freezing/thawing, and after hypotonic washing (described in Materials and Methods) as indicated.

vacuole, however, is very susceptible to lysis and permeabilization during hypotonic stress and the conditions used to perforate and remove soluble cytosolic protein might be expected to damage this organelle. The fate of the vacuole was determined under these conditions with soluble lumenal (mCPY and proteinase A) and integral membrane (alkaline phosphatase) marker protein analysis. Spheroplasts that were radiolabeled and chased long enough to contain mature vacuolar proteins in vivo (10-min pulse, 5-min chase), retained 40–50% of mCPY and mature proteinase A and 100% of alkaline phosphatase when they were taken through the perforation and washing regimen (data not shown). Thus, a subset of vacuoles either lysed or vesiculated, releasing lumenal contents while vacuolar membranes appeared to quantitatively remain associated with the perforated spheroplasts. These observations were consistent with morphological analysis of the perforated cells (see below).

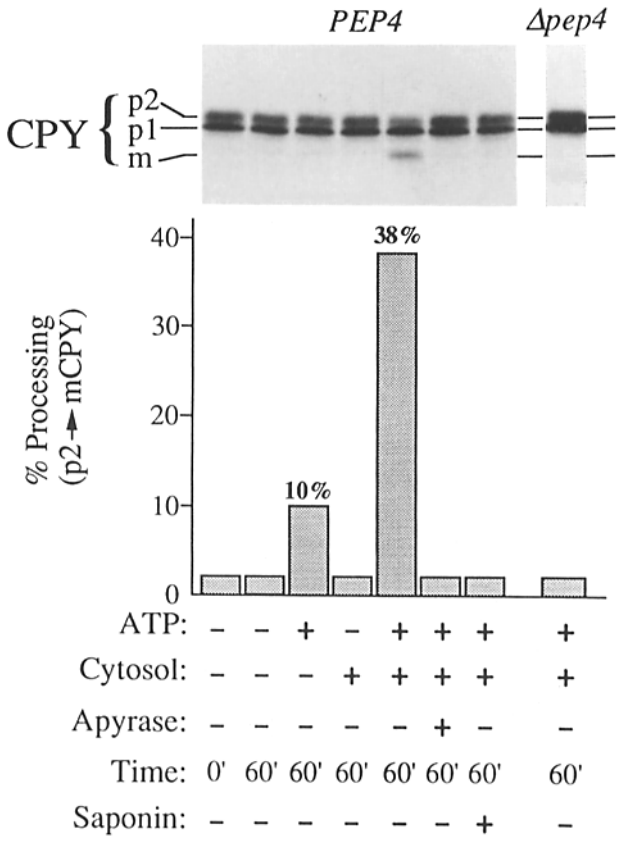

Figure 3. Energy and cytosol requirements for in vitro CPY maturation. Radiolabeled perforated spheroplasts from either *PEP4* or *Δpep4* stains were incubated with and without ATP (plus an ATP regeneration system), 150 μg of cytosolic protein, 50 U/ml apyrase, and saponin as indicated.

In Vitro CPY Maturation Requires ATP and Cytosolic Protein(s)

When intact spheroplasts containing radiolabeled p1 and p2CPY precursors were incubated with glucose at 25°C for 60 min before freezing, complete maturation of CPY took place (Fig. 2 *B*). Agents that inhibit energy production in the cell like azide and fluoride quantitatively blocked CPY maturation, even in the presence of glucose (Fig. 2 *B*). Depletion of ATP levels may raise the pH of the vacuole lumen potentially hindering efficient propeptide processing. However, this is very unlikely since neutralization of vacuolar pH with either the vacuolar H^+-ATPase inhibitor, bafilomycin A_1, (Banta et al., 1988) or in *Δvat2* (the structural gene for the 60-kD subunit of the vacuolar H^+-ATPase) yeast strains have no affect on the maturation of p2CPY (Yamashiro et al., 1990). Furthermore, inhibition of p2CPY processing with azide and fluoride was reversible after washing them from the cells (data not shown).

Freezing, thawing, and hypotonically washing yeast cells abolished their endogenous ability to transport CPY through the early secretory pathway en route to the vacuole. After just freezing and thawing, the cells still responded as if they were largely intact since glucose stimulated 60% maturation of the CPY precursors (Fig. 2 *B*). After washing with hypo-

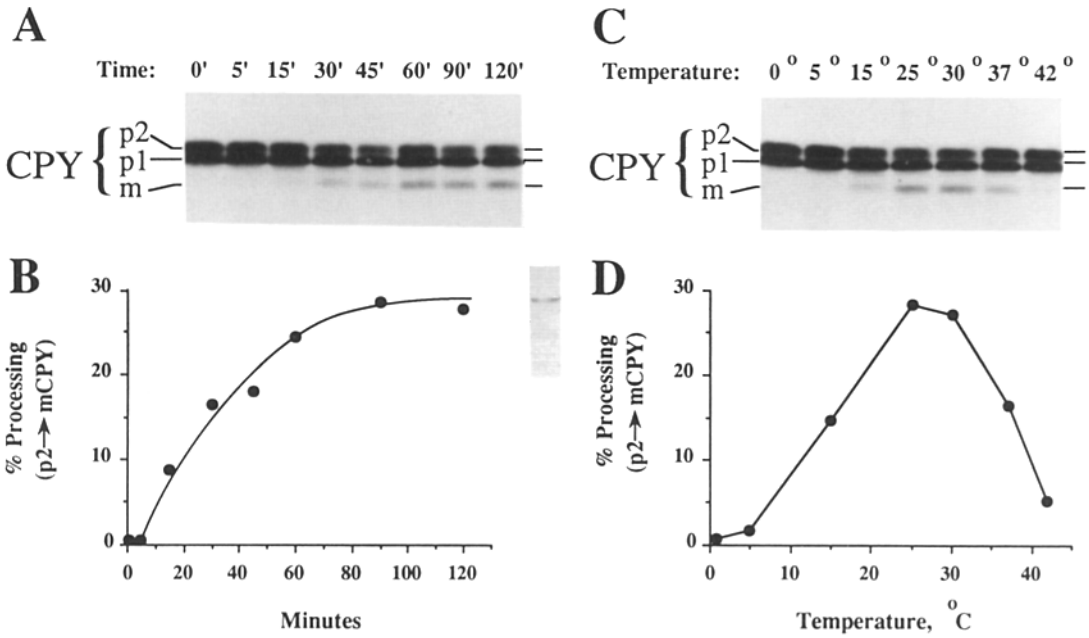

Figure 4. Time and temperature profiles of in vitro CPY maturation in perforated spheroplasts. Radiolabeled perforated spheroplasts, ATP, an ATP regeneration system, and cytosolic protein were mixed together at 0°C, then placed into aliquots, and incubated at 25°C for the indicated times (*A*) or at the indicated temperatures for 60 min (*C*). Quantitative analysis is shown for both the time (*B*) and temperature (*D*) profiles.

tonic buffers, however, 100% of CPY remained as the pl and p2 precursor forms (Fig. 2 *B*). These cells were quantitatively perforated, apparently losing the in vivo ability to convert glucose into the energy required to drive vesicular movement of organelle-to-organelle protein transport. Additionally, the cells may have also lost endogenous cytosolic protein factors that facilitate movement of the CPY precursors to the vacuole.

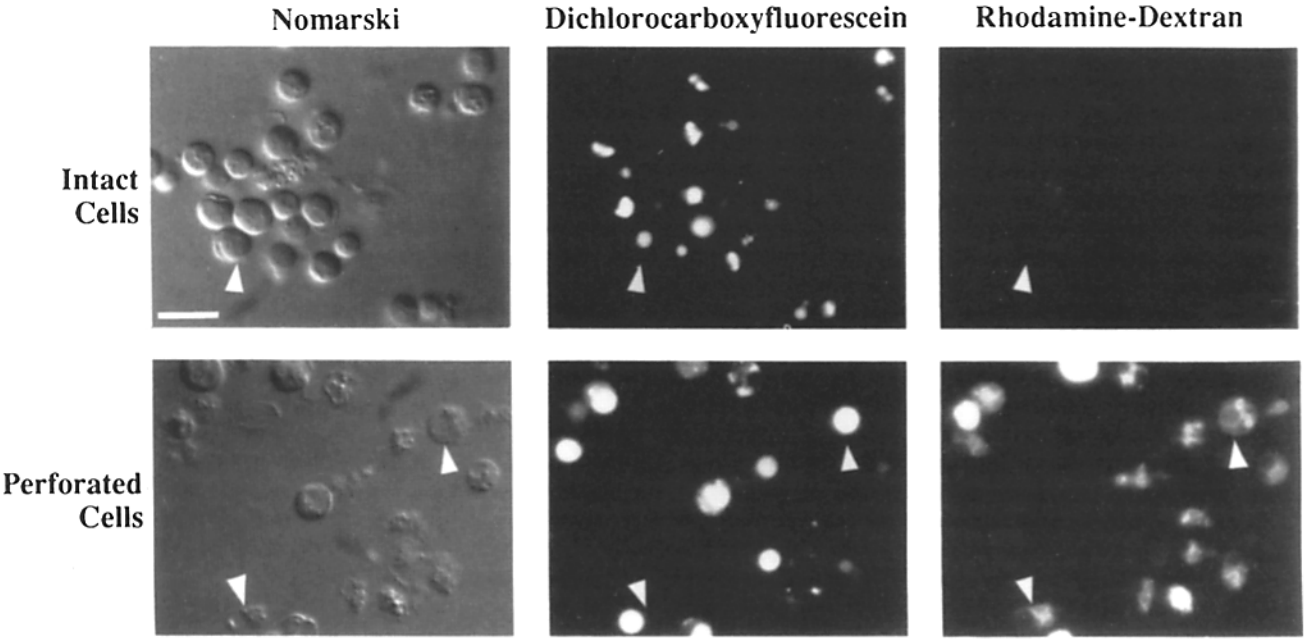

Figure 5. Vacuole morphology in perforated yeast cells. Yeast spheroplasts were stained at 30°C with 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (dichlorocarboxyfluorescein, 50 μM final) for 15 min in media containing 1.0 M sorbitol and buffered to pH 4.0 with 0.1 M citrate-KOH. The spheroplasts were then washed in isotonic buffer, frozen slowly over liquid N_2, and stored at −70°C until use. After thawing, an aliquot was removed, maintained in 1.0 M sorbitol, and counterstained with rhodamine-dextran (*Intact cells*). The remaining cells were washed with hypotonic buffers, incubated with ATP, an ATP regeneration system, and a cytosolic protein extract for 90 min at 25°C under identical conditions used for transport assays. After incubation, the perforated cells were counterstained with rhodamine-dextran, pelleted, and resuspended. Arrows show examples of intact or perforated cells with vacuolar fluorescence. Bar, 10 μm.

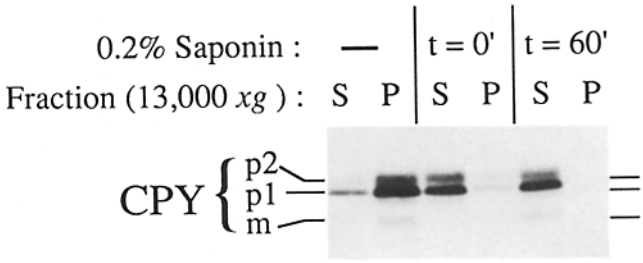

Figure 6. In vitro CPY maturation in perforated spheroplasts occurs within sealed membrane compartments. Three identical reaction mixtures containing perforated spheroplasts, an ATP regeneration system, and cytosolic protein were incubated at 25°C for 60 min. The cell suspensions were centrifuged for 2 min at 13,000 *g*. The supernatant (*S*) and pellet (*P*) fractions were immunoprecipitated for CPY. Saponin (0.2%) was added at the indicated times.

Successfully developing conditions that achieved nearly complete loss of cytoplasmic marker proteins and retention of lumenal ER and Golgi complex marker proteins provided the opportunity for reconstituting CPY maturation in vitro. As shown in Fig. 3, when the perforated spheroplasts were incubated with buffer alone at 25°C, the level of p1 and p2CPY did not change. However, when ATP (including an ATP regeneration system), and a cytosolic protein extract were added back to the perforated cell membranes, nearly 40% of p2CPY was converted to mCPY. Only 10% maturation of p2CPY took place when ATP and an ATP regeneration system alone were added back to the incubation. When densitometry was performed, the level of p1CPY did not decrease after incubation with either ATP alone or ATP plus cytosolic protein. Rather the level of p2CPY decreased in proportion to the increase in mCPY, which showed their expected precursor/product relationship. This precursor specificity continued throughout these studies and was the basis for reporting efficiency as the percentage of p2CPY processed to mCPY. When the cytosolic extract alone was added back, no detectable maturation took place, suggesting the extract had insufficient energy pools associated with it. An ATP depleting enzyme, apyrase, completely blocked maturation in the presence of ATP and cytosol. A nonhydrolyzable ATP analogue, adenylyl-imidodiphosphate (AMP-PNP), could not substitute for ATP and could competitively inhibit p2CPY processing when added in excess of ATP, suggesting that the reaction may require hydrolysis of ATP during incubation (data not shown). Thus, CPY processing in vitro appeared dependent on both exogenous energy and exogenous cytosolic protein(s). A specific protein factor(s) most likely caused the cytosol stimulation of in vitro CPY maturation in perforated spheroplasts. Heating the cytosolic extract at 95°C for 15 min destroyed its activity and a nonspecific protein (BSA) could not substitute for the cytosolic stimulation (data not shown). As expected, yeast strains containing a wild-type *PEP4* gene were capable of producing the observed p2CPY maturation. However, Δ*pep4* strains were completely deficient for in vitro processing, which demonstrates a requirement for proteinase A as is true in vivo (Fig. 3).

Characteristics of In Vitro CPY Maturation

The kinetics and temperature dependence of in vitro CPY maturation in perforated spheroplasts were analyzed to determine if their characteristics suggested a compartmentalized event. When reaction mixtures containing perforated spheroplast membranes, ATP, and a cytosolic extract were shifted from 0 to 25°C, a lag period of at least 5 min was observed in CPY maturation (Fig. 4 *A*). The same kinetic lag was also observed in vivo with intact cells after they were shifted from 0 to 25°C and incubated with glucose (data not shown). After the lag period, the in vitro reaction kinetics appeared linear from 15–60 min and reached a plateau between 1 and 2 h (Fig. 4 *B*). The reaction exhibited a temperature optimum of 25°C (Fig. 4, *C* and *D*). No mCPY was detected at 0 and 5°C, and very little maturation took place at 15, 37, and 42°C. The characteristics of this kinetic and temperature dependence data are consistent with a vesicle-mediated transport event (Balch et al., 1984; Goda and Pfeffer, 1988).

Vacuole Morphology in Perforated Yeast Spheroplasts

Marker protein analysis suggested that little damage to the ER and Golgi complex occurred during preparation of perforated yeast spheroplasts but the integrity of the vacuole, at least in part, was not maintained. However, the ability to reconstitute p2CPY maturation in vitro with exogenous ATP and cytosolic protein implied that a subset of vacuoles retained in vivo function and structure. To characterize vacuole morphology in perforated cells under these conditions, intact spheroplasts were first stained with dichlorocarboxyfluorescein, a fluorescent probe that is specifically se-

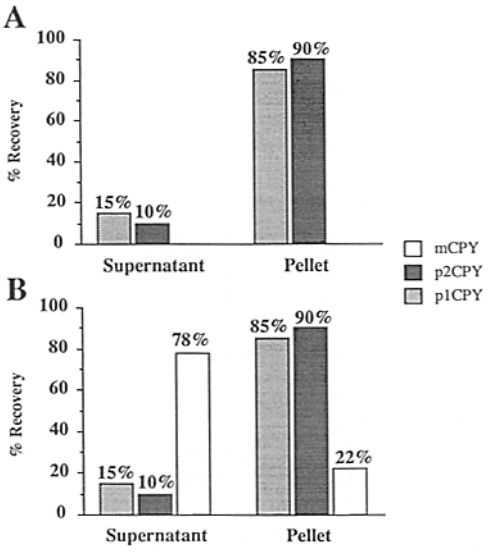

Figure 7. Differential fractionation of p2 and mCPY from perforated spheroplast membranes after hypotonic extraction. Two identical reaction mixtures containing perforated spheroplasts, ATP, an ATP regeneration system, and a cytosolic protein extract were incubated for 90 min at 0°C (*A*) or 25°C (*B*). After incubation, the cell suspensions were chilled on ice and fractionated as described in Materials and Methods. The supernatant and pellet fractions were immunoprecipitated for CPY and analyzed as described in Fig. 1. Processing efficiency (*B*) was 46%. As a control, when the perforated spheroplasts were treated with 0.2% saponin after incubation with energy and cytosolic protein, all three forms of CPY were quantitatively recovered in the supernatant.

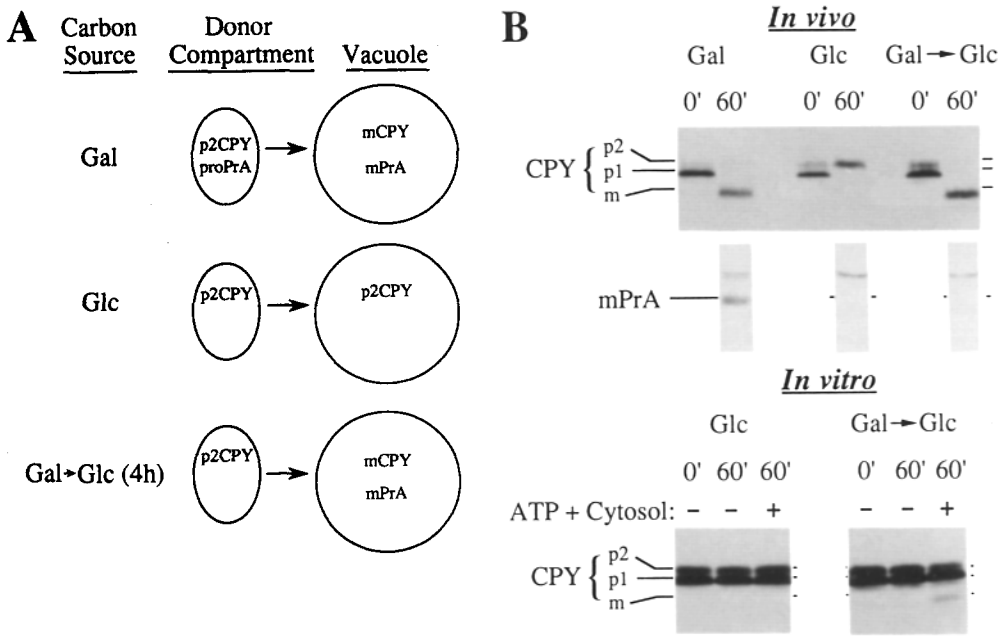

Figure 8. CPY maturation in *Δpep4* cells harboring a plasmid encoding *PEP4* under control of a galactose-inducible promoter. The yeast strain, DKY6224, was transformed with p*GAL1-PEP4* as described in Materials and Methods. (*A*) Schematic diagram depicting the compartmental consequences of CPY and PrA after induction, repression, and induction/repression of *PEP4* by growing the cells in galactose, glucose, or galactose followed by transfer to glucose, respectively. The identity of donor compartment could be a late Golgi cisterna, the *trans*-Golgi network, or an endosome-like organelle. (*B*) DKY6224 cells harboring p*GAL1-PEP4* were grown in galactose or glucose exclusively, and galactose followed by 4 h growth in glucose as indicated. The cells were converted to spheroplasts, pulse/chase-radiolabeled, washed three times in isotonic buffer, and perforated with freeze/thaw and hypotonic washing as described in Materials and Methods. Just before freezing, two aliquots of cells were removed from each culture condition, one serving as the zero time point, the other was incubated at 25°C for 60 min with galactose or glucose (5% each) as indicated (*In vivo*). The perforated cells were incubated with buffer alone or ATP, an ATP regeneration system, and 50 μg of cytosolic protein at 25°C for 60 min as indicated (*In vitro*). All reactions were immunoprecipitated for CPY. The 60-min time points from the in vivo analysis were immunoprecipitated for proteinase A (PrA). All immunoprecipitates were subjected to SDS-PAGE and fluorography.

questered within vacuoles (Pringle et al., 1989). The stained cells were then taken through the freeze/thaw and washing regimen used to perforate the plasma membrane and remove cytosolic proteins. They were then incubated at 25°C for 90 min with ATP and cytosolic protein. After this incubation the cells were counterstained with rhodamine-dextran (average molecular weight 70,000) to distinguish perforated from intact cells. After freezing and thawing, the prestained cells still contained sequestered dichlorocarboxyfluorescein and were impermeable to rhodamine-dextran (Fig. 5). This indicates, as stated previously, that freezing and thawing cells under these conditions does not result in significant breakage of the plasma membrane. However, after washing the thawed cells with hypotonic buffers, at least 90% were permeable to rhodamine-dextran (Fig. 5). More importantly, many of the permeable cells still contained vacuolar fluorescence, some with relatively enlarged vacuoles that remained stable and intact during a 90-min incubation with an energy source and cytosolic protein (Fig. 5).

In Vitro CPY Maturation Requires Sealed Membrane Compartments

Saponin was used to probe whether or not sealed organellar membranes were required for reconstitution of in vitro CPY processing. At appropriate concentrations, saponin does not solubilize membranes, rather it interacts with sterols forming 8–10-nm pores in the bilayer and creates leaky compartments (Dourmashkin et al., 1962; Glauert et al., 1962). Therefore, after treatment with saponin the soluble lumenal contents of organelles will no longer remain sequestered but will mix by random diffusion. If CPY maturation in perforated cells resulted from such mixing, then saponin treatment might stimulate the reaction. However, saponin completely blocked CPY maturation in the presence of ATP and cytosol (Fig. 2). At the concentration used, 0.2%, saponin does not solubilize integral membrane proteins found in two different organelles; vacuolar alkaline phosphatase (Klionsky and Emr, 1989) and the ER NADPH cytochrome-*c* reductase (Schauer et al., 1985).

Simple differential centrifugation procedures were used to further investigate saponin's effect on in vitro CPY maturation. When perforated cell membranes were subjected to centrifugation after incubation without saponin, very little p1 or p2CPY, and no detectable mCPY fractionated with the supernatant (Fig. 6). Conversely, when saponin was added to the perforated cell membranes after incubation, p1, p2, and mCPY quantitatively fractioned with the supernatant (Fig. 6). Saponin's effect on in vitro CPY maturation strongly suggested that before and after incubation CPY remained within sealed membrane compartments, arguing against the possibility that p2CPY processing took place in a freely diffusible manner.

Differences in osmotic stability of the vacuole versus the ER and Golgi complex were examined to determine if the observed p2CPY processing resulted from intercompartmental transport. If the conversion of p2 to mCPY were occurring in the same sealed membrane compartment, then the two species should fractionate similarly with differential centrifugation before or after incubation. Under certain osmotic stress conditions the vacuole tends to lyse, expelling its con-

tents as mCPY (or p2CPY in a *pep4* strain) always fractionates like a soluble protein once the vacuolar membrane is disrupted (Ohsumi and Anraku, 1981; Mechler et al., 1987; Eakle et al., 1988). In contrast, the ER and Golgi complex tend to retain their lumenal contents rather than lysing when subjected to hypotonic stress (Esmon et al., 1981; Eakle et al., 1988). To determine where p2 and mCPY fractionated, perforated spheroplast membranes were pelleted after incubation in the presence of ATP and cytosol, resuspended in a hypotonic extraction buffer, agitated vigorously, and then subjected to differential centrifugation. As shown in Fig. 7, when incubated at 0°C, conditions where maturation did not take place, 85% of pl, and 90% of p2CPY were recovered in the pellet. In contrast, after incubation at 25°C nearly 80% of the mCPY was recovered in the supernatant fraction and 90% of the remaining p2CPY was still recovered in the pellet. Additional experiments showed that 100% of the released mCPY remained soluble after further fractionation of the 13,000-*g* supernatant with centrifugation at 100,000 *g*. These results suggest that incubation of perforated cell membranes with ATP and cytosol promotes transfer of p2CPY from an osmotically stable compartment to an osmotically sensitive compartment, possibly the vacuole.

In Vitro CPY Maturation Is Dependent on Intercompartmental Transport

The above data and previous studies concerning CPY maturation in vivo (Stevens et al., 1982; Klionsky et al., 1988) can support two generalized models for in vitro reconstitution of CPY maturation in perforated spheroplasts. In the first model, p2CPY could reside in a distinct donor compartment at the time of initiating incubation with energy and cytosolic protein. During incubation, transport vesicles would bud away from this compartment, move to an acceptor compartment (presumably the vacuole), fuse, and deliver p2CPY for propeptide processing. This model predicts that propeptide processing is dependent on a vesicle-mediated intercompartmental transport event. In the second model, during incubation with energy and cytosolic protein, a compartment containing p2CPY along with the zymogen forms of proteinase A and proteinase B, may undergo a change in its lumenal environment (i.e., pH or ionic strength). Activation of the zymogen forms of proteinases A and B may then take place, allowing for propeptide cleavage from p2CPY. Because all three proteins are likely to share a common pathway during their transit to the vacuole as zymogen precursors, intracompartmental autoactivation and propeptide processing was a concern. The fractionation differences between p2 and mCPY after hypotonic stress would have masked intracompartmental maturation if a physical change in the compartment also took place during incubation causing it to become osmotically labile (Fig. 7).

To experimentally distinguish between these two models, conditions for a donor–acceptor complementation assay that demanded intercompartmental transfer for propeptide processing of p2CPY needed to be set up. Placing the *PEP4* gene under control of the galactose-inducible *GAL1* promoter in a Δ*pep4* strain allowed for conditions that would deplete the p2CPY donor compartment of proteinase A, thereby allowing us to distinguish between intra- and intercompartmental p2CPY processing. Earlier, Δ*pep4* yeast strains were shown to be absolutely defective for reconstituting p2CPY maturation in perforated cells (Fig. 3). Vacuole delivery of p2CPY is not dependent on the presence of active proteinase A because Pep4⁻ strains contain vacuoles that accumulate p2CPY (Stevens et al., 1982). When Δ*pep4* strains harboring a $GAL1_{pr}$- *PEP4* plasmid are grown exclusively in galactose as the carbon source, the cells should be phenotypically Pep4⁺ (Fig. 8 *A*). This was observed experimentally as growth in galactose produced a Pep4⁺ phenotype not only because CPY was completely matured after a 1-h chase, but also because radiolabeled proteinase A was detected by immunoprecipitation (Fig. 8 *B*; *In vivo*). Conversely, growth exclusively in glucose, a repressor of the $GAL1_{pr}$, eliminated *PEP4* gene expression such that no radiolabeled proteinase A could be detected and p2CPY was not matured even after a long chase period (Fig. 8 *B*; *In vitro*). The slow turnover and stable inheritance of proteinase A across many cell divisions (Jones, 1977; and unpublished observations) allowed for the following regulated expression scenario. After induction of *PEP4* expression with growth in galactose, the cells could be shifted to glucose long enough for complete repression and would no longer biosynthesize proteinase A. The proteinase A that was made during induction in galactose would be predicted to reside in its steady-state compartment, the vacuole. Therefore, control of *PEP4* gene expression sets up an intracellular donor/acceptor compartmentalization since all vacuolar precursor compartments containing p2CPY could be purged free of zymogen pools of proteinase A, demanding intercompartmental transport for processing to take place (Fig. 8 *A*).

The fate of CPY was determined after induction and repression of *PEP4* gene expression with pulse–chase analysis in vivo before testing the effect in vitro. Preliminary experiments indicated that after shifting from galactose to glucose media for 1 h, proteinase A synthesis was repressed ~100-fold, and completely repressed (>1,000-fold) 3–4 h after shifting to glucose media. Spheroplasts radiolabeled after shifting from galactose to glucose media for 4 h contained significant pools of pl and p2CPY that, during a 60-min chase, were completely converted to mCPY similar to when the cells were grown exclusively in galactose (Fig. 8 *B*; *In vivo*). As expected, radiolabeled proteinase A was not detected in these cells (Fig. 8 *B*; *In vivo*). When these same Δ*pep4* cells were taken through the perforation procedure and incubated with ATP and cytosolic protein, 20% of p2CPY was matured after glucose repression of proteinase A biosynthesis (Fig. 8 *B*; *In vitro*). As a negative control, growth exclusively in glucose gave the expected result, no maturation of p2CPY (Fig. 8 *B*; *In vitro*). The half-time for proteinase A maturation is ~6 min (Klionsky et al., 1988), thus, the 4 h glucose shift for repression of *PEP4* should be more than sufficient to purge biosynthetic pools and reach a steady-state compartmentalization of active proteinase A. Therefore, these results indicated that in vitro CPY maturation required intercompartmental transfer of p2CPY before propeptide cleavage, which was independent of biosynthetic proteinase A pools.

Maturation of CPY during regulated *PEP4* gene expression indicated that the acceptor compartment for p2CPY transport in vitro was the vacuole, which was analyzed further with Ficoll step-gradient centrifugation. Flotation of cell lysates from the bottom to the top of a single Ficoll gra-

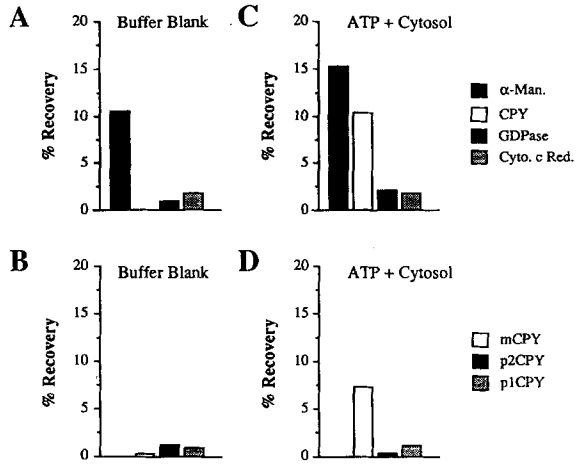

Figure 9. Ficoll step-gradient fractionation of perforated cell membranes. Four incubations with unlabeled (*A* and *C*) and radiolabeled (*B* and *D*) perforated spheroplasts containing ATP, an ATP regeneration system, and cytosolic protein (*C* and *D*) or buffer alone (*A* and *B*) were done in parallel for 90 min at 25°C. After incubation, the perforated spheroplasts were pelleted, resuspended in lysis buffer, loaded at the bottom of a multi-step Ficoll gradient as described in detail in Materials and Methods. 10 0.5-ml fractions were collected from the top to the bottom of all gradients. Marker enzymes were assayed from the gradients containing unlabeled cells, and immunoprecipitations for CPY were done from the gradients containing labeled cells. The vacuole-enriched fraction represents the uppermost fraction, which contains the 0–4% Ficoll interface. Total recoveries of enzymes throughout the unlabeled gradient (*C*) were 100% for α-mannosidase (vacuole membrane); 74% for cytochrome-*c* reductase (ER membrane); 89% for GDPase (Golgi lumen); and 80% for CPY (vacuole lumen). CPY activity was not assayed in the unlabeled gradient (*A*).

dient is sufficient to enrich for vacuolar enzymes from yeast spheroplasts. The overall recovery of vacuoles varies with different lysis conditions and different strains (Wiemken et al., 1979; Ohsumi and Anraku, 1981; Stevens et al., 1982). Perforated cells presented a problem in vacuole isolation as integrity of the plasma membrane was already compromised, removing osmotic turgor, which made DEAE-dextran-mediated lysis (Wiemken et al., 1979; Johnson et al., 1987) unsuccessful at dispersing organelles. Therefore, triethanolamine-based buffers (Makarow, 1985; Walworth et al., 1989) were used with modifications in lysis and gradient conditions so that vacuoles could be floated from perforated cells (described in Materials and Methods). After incubation of nonlabeled perforated spheroplast membranes with ATP and cytosolic protein, 15% of α-mannosidase and 10% of CPY enzyme activity were recovered in the top of the Ficoll gradient (Fig. 9). In contrast to these vacuolar enzyme markers, the ER and Golgi complex markers, NADPH cytochrome-*c* reductase (Opheim, 1978) and guanosine diphosphatase (Abeijon et al., 1989), respectively, were not recovered in this fraction. Likewise, when radiolabeled perforated spheroplast membranes were subjected to this fractionation in parallel, after incubation with energy and cytosolic protein, pl and p2CPY were not enriched in the vacuole fraction (Fig. 9). However, mCPY was enriched 17-fold over p2CPY in the top of the gradient; the same fraction that α-mannosidase and CPY enzyme activities were recovered. For complete analysis, ten equal fractions were collected from the top to the bottom of the entire gradient and 35–50% of pl and p2CPY were recovered in regions below the vacuole-enriched layer and above the lysate layer, while the remaining pl, p2, and mCPY were recovered in the lysate layer. Furthermore, under conditions where in vitro CPY maturation did not occur (incubation with buffer alone at 25°C), p2CPY was not recovered in the top of the gradient (Fig. 9 *B*). Thus, direct subcellular isolation of intact vacuoles from perforated cells provides more definitive evidence to suggest that intercompartmental transport of p2CPY from a donor compartment to an acceptor compartment, the vacuole, has been reconstituted in vitro.

vps Mutants Are Defective for CPY Maturation In Vitro

A major impetus for initiating this reconstitution was not only to address biochemical aspects of intercompartmental protein transport to the vacuole, but also to analyze and characterize *vps* mutants. Three different *vps* mutants (*vps15*, *33*, and *34*) were radiolabeled and converted to perforated spheroplasts. Each mutant was found to be completely defective for intercompartmental p2CPY maturation, indicating that the in vitro reconstitution is dependent on *VPS* gene product function (data not shown). Wild-type cytosolic protein extracts were unable to biochemically complement this maturation defect. A trivial explanation for this result could be that the defect in these mutants was not associated with the cytosol. Subcellular fractionation indicates that this may be the case for *VPS15p* (Herman et al., 1991) and *VPS34p* (Herman and Emr, 1990). Another more subtle explanation for failure to observe processing in these mutants was that they mislocalized vacuolar hydrolases to such an extent that their vacuoles lacked active proteinase A and acted as functionally silent acceptors. Unfortunately, all alleles of the *vps* mutants isolated thus far exhibit vacuolar protein sorting defects at all temperatures. Temperature-sensitive alleles of certain cloned *VPS* genes that exhibit a conditional protein sorting defect are currently being isolated, which will enable us to better analyze their function by carrying out in vitro reconstitution assays at the permissive and nonpermissive temperature.

Discussion

We have reconstituted intercompartmental transport and proteolytic conversion of a precursor form of CPY, p2CPY, to the mature form of this enzyme. Some basic biochemical requirements of this cellular process have been elucidated in vitro, which provides the opportunity for a more detailed understanding of the molecular mechanisms that facilitate vacuolar protein delivery in yeast. An authentic in vitro event should closely mimic the characteristics observed for that event in vivo, yet be amenable to reconstituted biochemical analysis. Four criteria were met with this in vitro reconstitution of CPY protein transport to the vacuole. First, the process was shown to be energy dependent; second, it required the presence of cytosolic protein factor(s); third, it showed precursor specificity; and fourth, it was intercompartmental. These properties, common to all interorganellar vesicle-mediated transport events, have now been demon-

strated for transport and maturation of a yeast vacuolar protein.

Permeabilization of the yeast plasma membrane to produce perforated cells was required for successful biochemical reconstitution of yeast vacuolar protein delivery. Perforated cell systems maintain the spatial and functional organization of intracellular organelles. These systems have been used for the reconstitution of membrane transport between the ER and early Golgi complex in mammalian (Beckers et al., 1987; Simons and Virta, 1987) and yeast cells (Baker et al., 1988; Ruohola et al., 1988), and between endosomes and the *trans*-Golgi network in mammalian cells (Goda and Pfeffer, 1989). Using perforated cells for reconstitution purposes requires selective removal of cytosolic components while intracellular membrane-sealed organelles are retained within a permeable plasma membrane. Successfully achieving this in yeast cells for reconstituting intercompartmental p2CPY maturation required different conditions than those used previously for reconstituting ER to early Golgi transport (Baker et al., 1988; Ruohola et al., 1988). Initially, a large percentage of cells resisted various perforation regimens and always exhibited a latent ability to mature CPY, which resulted in significant background when incubations with buffer alone were performed. However, thorough hypotonic washing conditions were introduced to prevent this, such that even incubation with glucose could not stimulate CPY maturation in perforated cell preparations. This effect functionally demonstrated that quantitative permeabilization took place.

Efficient p2CPY maturation was only observed in the presence of ATP, an ATP regeneration system, and cytosolic protein extracts. Incubation with cytosolic extracts alone consistently failed to produce mCPY. Steady-state concentrations between 0.1 and 1 mM ATP, maintained by a regeneration system, are required for efficient in vitro CPY maturation. Supplementing the reaction with up to 5 mM ATP (without a regeneration system) and cytosol produces little response, suggesting that this crude reconstituted system can consume ATP before it is used for intercompartmental transport (unpublished results). The 10% conversion of p2CPY that occurred in the presence of just ATP plus a regeneration system (Fig. 3) most likely reflected the activity of residual cytosolic protein, in trace amounts, that was not washed free from the perforated cells. More rigorous washing conditions used in recent experiments support this conclusion since the p2CPY processing stimulated by ATP alone has dropped from the 10% reported here to 1–3%. With these more thoroughly washed perforated cell membranes, exogenously added cytosolic protein extracts can stimulate p2CPY maturation some 10–20-fold (unpublished results).

A specific protein factor(s) causes the cytosol stimulation of in vitro CPY maturation in perforated cells. Although formal proof requires purification of this putative factor(s), the cytosolic activity behaves like a protein. Heating the cytosolic extract at 95°C for 15 min destroys its ability to stimulate ATP-dependent CPY maturation. More convincingly, the activity in cytosolic extracts fractionates with ammonium sulfate precipitation, withstands desalting columns, and partially resolves with gel filtration chromatography (unpublished results). These characteristics argue against the possibility that small molecular weight cytosolic components like Ca^{2+} or nucleotides give rise to stimulation of ATP-dependent in vitro CPY maturation. Future efforts will focus on purification and characterization of the cytosolic protein factor(s) that are required to assay CPY maturation in perforated cells.

An important aspect of intercompartmental protein transport through the secretory pathway is donor–acceptor compartment specificity. Reconstituting various interorganellar steps with perforated cells in vitro usually takes advantage of knowing what donor compartment a given protein substrate resides in before incubation. The use of a mutant protein (Beckers et al., 1987; Simons and Virta, 1987), mutant cells (Goda and Pfeffer, 1989), or specific accumulation within a known donor compartment (Baker et al., 1988; Ruohola, et al., 1988) has been instrumental in previous reconstitutions. Radiolabeling wild-type yeast cells in vivo has the advantage of being an efficient method to generate the Golgi-modified p2CPY precursor needed for this in vitro reconstitution. A disadvantage to this approach is not knowing specifically in what compartment(s) p2CPY is trapped. The use of *sec* mutants to block organelle passage of CPY at known steps in the early secretory pathway is not feasible because no *sec* mutant specifically accumulates p2CPY, the direct precursor to mCPY (Stevens et al., 1982; Franzusoff and Schekman, 1989). Significant amounts of radiolabeled p1CPY in the perforated spheroplasts allowed us to assess the specificity of in vitro maturation. Aberrant maturation of p1CPY might have been expected to occur in vitro from potential nonspecific bulk aggregation and fusion of different organellar membranes. However, this was never observed; the level of p1CPY did not change, whereas p2CPY was exclusively converted to mCPY. Furthermore, conversion of p1 to p2CPY also was not observed, suggesting that reconstitution of protein transport between the ER and Golgi complex for CPY was either not occurring or not detectable under these conditions. Acquisition of α1,6-linked mannose residues on pro-α-factor is used to monitor in vitro transport from the ER to the early Golgi in yeast (Baker et al., 1988; Ruohola et al., 1988). Electrophoretic resolution of p2 from p1CPY requires addition of α1,2-linked and/or α1,3-linked mannose residues, modifications that take place in later Golgi compartment(s) (Fransuzoff and Schekman, 1989). Thus, transport of p1CPY from the ER to the early Golgi complex would be difficult to detect on the basis of an electrophoretic mobility shift.

The p2CPY precursor specificity of the reconstituted transport assay suggests that compartmental specificity also exists. The characteristics of p2CPY processing in perforated cells indicate that this event results from transfer of p2CPY between specific donor and acceptor compartments. First, the strict dependence on ATP and cytosol follows characteristics of all known intercompartmental transport reactions to date. Second, all forms of CPY remain sedimentable both before and after incubation under conditions that maintain sealed membrane barriers but not after saponin permeabilization. Since saponin inhibits p2CPY processing, lumenal contents of organelles need to be sequestered for reconstitution, which apparently cannot efficiently take place with freely diffusible components under these conditions. Third, after incubation, mCPY fractionates into a supernatant while its precursor, p2CPY remains in a pellet after hypotonic extraction. Before incubation, p2CPY continues to fractionate with a pellet. Therefore, mCPY shows character-

istics of being enclosed in an osmotically labile compartment like the vacuole. Fourth, and most convincingly, maturation of CPY still took place in vitro, even after first induction and then complete repression of *PEP4* gene expression. Regulating *PEP4* expression under control of the galactose-inducible promoter, *GAL1*, reveals that zymogen pools of proteinase A are not required for efficient compartmentalized p2CPY maturation. The results from this regulated expression would be misleading if repression of *PEP4* was incomplete after induction or if the turnover rate for proteinase A was rapid. However, after 4 h of repression, radiolabeled proteinase A cannot be detected by immunoprecipitation, even with excessively long autoradiography, and processing of CPY is normal even after repression times as long as 15–20 h (unpublished results). Therefore, having active processing proteases localized to their steady-state compartment is both necessary and sufficient for efficient propeptide cleavage from p2CPY. Altogether, the data support that reconstitution of p2CPY maturation in perforated cells reflects an authentic transfer from a donor compartment to an acceptor compartment, the vacuole.

The donor compartment involved in delivering p2CPY to the vacuole is difficult to precisely define. The limited carbohydrate additions that take place on CPY as it traverses through the yeast Golgi complex prevent detailed knowledge regarding its specific cisternal location. Recent evidence suggests that the early Golgi form of CPY cannot be resolved from the ER form, plCPY, with SDS-PAGE. It may not be until the addition of α1,3 mannose linkages in later Golgi cisternae that p2CPY becomes apparent (Fransuzoff and Schekman, 1989). This would suggest that the identity of the specific donor compartment is a late Golgi cisterna or perhaps *Saccharomyces cerevisiae's* equivalent to the mammalian *trans*-Golgi network (TGN; Griffiths and Simons, 1986). Available evidence does not implicate an endosome in the pathway between the yeast Golgi complex and vacuole like the established role of this organelle in mammalian lysosomal protein delivery (von Figura and Hasilik, 1986; Mellman et al., 1986). However, the possibility that the in vitro reconstitution has uncovered some other stable post-Golgi complex, prevacuole donor compartment such as an endosome cannot be ruled out. To distinguish between a Golgi complex–associated and a putative endosome-like donor compartment for p2CPY requires new fractionation methods and marker enzyme assays (Singer and Reizman, 1990).

The intercompartmental transport of p2CPY in vitro is relatively efficient with as much as 45% conversion in some experiments. Activity of cytosolic protein(s) may affect the efficiency since some cytosol preparations are more potent than others at stimulating ATP-dependent maturation of p2CPY. Donor compartment specificity may also affect the efficiency of in vitro reconstitution. Pools of p2CPY probably reside not only in the donor compartment that directly precedes the vacuole, but also in other, earlier compartment(s) of the vacuolar delivery pathway. For instance, if the direct donor compartment is truly analogous to the mammalian TGN, then late cisternae of the Golgi complex proper could also house unprocessed p2CPY. This hypothesis suggests that p2CPY transits through more than one compartment before arriving at the vacuole and the final transport step may be more amenable to reconstitution. Functional integrity of the vacuole acceptor compartment also is likely to play an important role in efficient reconstitution of intercompartmental p2CPY transport. The hypotonic wash conditions that remove >95% of cytosolic protein from yeast cells also remove, on average, 50–60% of soluble lumenal vacuolar proteins. The vacuolar membrane, however, is completely contained in the perforated cells suggesting that a subset of vacuoles become permeabilized and lose their lumenal contents. Thus, some cells within the population may contain functionally silent acceptor vacuoles where processing would be undetectable. Morphological examination corroborates this conclusion since some perforated cells (25–50%) appear to have lost previously sequestered vacuolar fluorescence and do not have discernable vacuoles when viewed with Nomarski optics (Fig. 5).

This reconstituted perforated cell system is designed for biochemical manipulation of vacuolar protein delivery in an environment that duplicates in vivo events as close as possible. The collection of >40 complementation groups of *vps* mutants with defects in sorting, transport, or delivery of vacuole protein precursors potentially ensures the ability to validate reconstitution of physiologically relevant processes. Defective membrane or soluble extracts from *vps* mutants in biochemical complementation analyses could reveal information regarding potential involvement and subcellular location of *VPS* gene product function(s) in the vacuolar protein delivery pathway. Consistent with this, we found that perforated *vps15*, *33*, and *34* cells were defective for in vitro p2CPY maturation. Wild-type cytosolic extracts were insufficient at rescuing the vacuolar protein sorting defects in these mutants. In general, this suggests that successful in vitro reconstitution of intercompartmental transport to the vacuole directly or indirectly may require function of these *VPS* gene products. For *vps15* and *34*, the inability to rescue perforated mutant cells with wild-type cytosolic extracts is expected since *VPS15*p and *VPS34*p are not washed away from wild-type perforated cells (unpublished results). Defects in these mutants are most likely associated with the perforated cell membranes rather than the cytosol and to biochemically complement these mutants will require more than just a wild-type soluble protein extract. Furthermore, cytosolic extracts derived from *vps15* and *34* show no defects when used with wild-type perforated cells, which corroborates this conclusion (unpublished results). Likewise, subcellular location of the *vps33* defect may not be limited to the cytosol. Although *VPS33*p is a soluble cytoplasmic protein (Banta et al., 1990), wild-type cytosolic extracts cannot restore p2CPY maturation in vitro. Currently, protein extracts derived from membrane fractions are being tested for the ability to biochemically complement the above mentioned mutants since the addition of soluble protein has no effect.

Biochemical reconstitution of specific steps in complex multi-step cellular pathways like yeast vacuolar protein delivery offers the ability to assay those components that directly facilitate the reconstituted step. Many *VPS* gene products presumably function in a specific ordered pathway that would involve various steps like sorting vacuolar proteins in the Golgi lumen; specific vesicle formation from the Golgi membrane; vesicular transport from the Golgi complex (and possibly through later Golgi compartments) to the vacuolar membrane; and membrane fusion of transport vesi-

cles for final vacuolar delivery. Only those *vps* mutants that directly affect the step(s) being reconstituted will be amenable to in vitro analysis. Screening through the entire collection of *vps* mutants for possible cytosolic defects is under way. Additionally, isolating conditional *vps* mutants that exhibit defects in a temperature-sensitive manner will also facilitate biochemical complementation analysis. We anticipate that the combined use of genetic, and now biochemical, approaches will help elucidate further details of the molecular mechanism(s) used to accurately sort, transport, and deliver vacuolar precursor proteins through the late secretory pathway.

We thank David Baker and Randy Schekman (University of California, Berkley) for communicating results before publication. We are also grateful to Jeremy Thorner for antiserum against phosphoglycerol kinase and to the members of the Emr laboratory for useful discussions and comments on the manuscript.

This work was supported by Public Health Service grant GM-32703 from the National Institutes of Health. T. A. Vida and T. R. Graham were both supported by Postdoctoral Research Fellowships from the American Cancer Society.

Received for publication 20 June 1990 and in revised form 16 August 1990.

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