

Yeast Vacuolar Proenzymes Are Sorted in the Late Golgi Complex and Transported to the Vacuole via a Prevacuolar Endosome-like Compartment

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Abstract. We are studying intercompartmental protein transport to the yeast lysosome-like vacuole with a reconstitution assay using permeabilized spheroplasts that measures, in an ATP and cytosol dependent reaction, vacuolar delivery and proteolytic maturation of the Golgi-modified precursor forms of vacuolar hydrolases like carboxypeptidase Y (CPY). To identify the potential donor compartment in this assay, we used subcellular fractionation procedures that have uncovered a novel membrane-enclosed prevacuolar transport intermediate. Differential centrifugation was used to separate permeabilized spheroplasts into 15K and 150K *g* membrane pellets. Centrifugation of these pellets to equilibrium on sucrose density gradients separated vacuolar and Golgi complex marker enzymes into light and dense fractions, respectively. When the Golgi-modified precursor form of CPY (p2CPY) was examined (after a 5-min pulse, 30-s chase), as much as 30–40% fractionated with an intermediate density between both the vacuole and the Golgi complex. Pulse-chase labeling and fractionation of membranes indicated that p2CPY in this gradient region had already passed through the Golgi complex, which kinetically ordered it between the Golgi and the

vacuole. A mutant CPY protein that lacks a functional vacuolar sorting signal was detected in Golgi fractions but not in the intermediate compartment indicating that this corresponds to a post-sorting compartment. Based on the low transport efficiency of the mutant CPY protein in vitro (decreased by sevenfold), this intermediate organelle most likely represents the donor compartment in our reconstitution assay. This organelle is not likely to be a transport vesicle intermediate because EM analysis indicates enrichment of 250–400 nm compartments and internalization of surface-bound ³⁵S- α -factor at 15°C resulted in its apparent cofractionation with wild-type p2CPY, indicating an endosome-like compartment (Singer, B., and H. Reizman. 1990. *J. Cell Biol.* 110:1911–1922). Fractionation of p2CPY accumulated in the temperature sensitive *vps15* mutant revealed that the *vps15* transport block did not occur in the endosome-like compartment but rather in the late Golgi complex, presumably the site of CPY sorting. Therefore, as seen in mammalian cells, yeast CPY is sorted away from secretory proteins in the late Golgi and transits to the vacuole via a distinct endosome-like intermediate.

PROTEIN transport within the eukaryotic secretory pathway involves the intercompartmental delivery of proteins from the ER to the cell surface via the Golgi complex (Palade, 1975). The compartmental framework of the Golgi complex includes the *cis* Golgi network, medial Golgi cisternae, and the TGN, which are topologically and functionally distinct (Mellman and Simons, 1992). Carrier vesicles facilitate transport from the ER, to and through the Golgi compartments, to the TGN, where selection and sorting occurs for intracellular and extracellular destinations (Griffiths and Simons, 1986).

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Saccharomyces cerevisiae mutants and biochemical reconstitution assays in mammalian systems have greatly enhanced our understanding of the discrete vesicle-mediated steps between various secretory pathway organelles (for reviews see Rothman and Orci, 1992; and Pryer et al., 1992). The temperature-sensitive *sec* mutants not only identified a variety of gene products required for movement of secretory proteins to the cell surface (Novick et al., 1980), but also determined their epistatic relationships, which confirmed the order of events within the eukaryotic secretory pathway (Novick et al., 1981; Kaiser and Schekman, 1990). Cell-free biochemical assays, measuring transfer between membrane-enclosed donor and acceptor compartments, have also played a role in elucidating details of vesicle-mediated trans-

port (Balch, 1989). This approach has identified several proteins acting as transport factors such as the *N*-ethylmaleimide sensitive fusion factor, NSF, (Block et al., 1988), numerous small molecular weight G proteins (Balch, 1990; Pfeffer, 1992), several soluble NSF attachment proteins, SNAPs, (Clary et al., 1990; Clary and Rothman, 1990), EFTf1, a protein involved in recycling from late endosomes to the TGN (Goda and Pfeffer, 1991), and a 145-kD protein that acts during Ca²⁺-regulated secretion in neuroendocrine cells (Walent et al., 1992). The genetic and biochemical approaches studying protein transport within the early secretory pathway have been highly complementary as several *SEC* gene products are homologous to mammalian proteins identified with cell-free assays.

Genetics and biochemistry are also being combined to dissect a branch point in the yeast secretory pathway, sorting and transport to the lysosome-like vacuole. The vacuole protein sorting defective mutants, *vps*, fall into over 40 complementation groups whose principle phenotype is the secretion of vacuolar precursor proteins (Bankaitis et al., 1986; Rothman and Stevens, 1986; Robinson et al., 1988; Rothman et al., 1989). Specific modifications, which mark transit through secretory organelles, occurs for most precursor forms of vacuolar proteins like carboxypeptidase Y (CPY).¹ The 67 kD p1CPY precursor has four N-linked core oligosaccharides and resides in the ER and early Golgi complex (Stevens et al., 1982; Franzusoff and Schekman, 1989). The 69-kD p2CPY precursor contains additional mannose residues, predominately α 1,3-linked, and resides in later compartments of the Golgi complex (Franzusoff and Schekman, 1989; Graham and Emr, 1991). Upon arrival in the vacuole, the 90 aa propeptide on p2CPY is proteolytically removed, which gives rise to the 61-kD mature form of CPY (mCPY; Stevens et al., 1982). All of the *vps* mutants secrete the Golgi-modified p2CPY precursor to the cell surface. A biochemical assay that faithfully reconstitutes intercompartmental transport and proteolytic maturation of CPY has recently been developed (Vida et al., 1990). In addition to being ATP and cytosol dependent, the in vitro assay shows *VPS* gene product-dependence and specificity for p2CPY. The specificity for p2CPY indicates that the assay shows preference for a specific donor compartment, which could be the Golgi complex. The basic compartmental transport pathway for CPY (ER → Golgi → vacuole) (Stevens et al., 1982) is now more completely understood with recent evidence demonstrating the existence of yeast Golgi subcompartments (Cunningham and Wickner, 1989; Franzusoff and Schekman, 1989; Bowser and Novick, 1991; Graham and Emr, 1991). Indeed, p2CPY travels through the Kex2p-containing late Golgi compartment, which is the presumptive site for sorting vacuolar proteins away from secreted proteins (Graham and Emr, 1991). However, even with this more highly refined view, little is known about the steps between the late Golgi and the vacuole.

We have characterized the pathway between the late Golgi and the vacuole using subcellular fractionation techniques. The results demonstrate that a novel prevacuolar compartment exists between the late Golgi and the vacuole. This

compartment is not likely to be a transport vesicle, but rather is endosome-like because internalized α -factor colocalizes with it. The prevacuolar, endosomal compartment appears to act as the donor organelle during in vitro reconstitution of intercompartmental transport to the vacuole. Additionally, vacuolar proteins are not sorted in this compartment but rather must be sorted to this compartment before they reach the vacuole. Overall, the data suggest that yeast vacuolar protein traffic is remarkably similar to mammalian lysosomal protein traffic: each appears to require transit through an endosomal intermediate compartment.

Materials and Methods

Strains and Plasmids

The wild-type parental strain for these studies was SEY6210 *MAT α* ; *ura3-52*; *his3- Δ 200*; *trp1- Δ 901*; *leu2-3,112*; *lys2-801*; *suc2- Δ 9*; *GAL*. SEY6210 was transformed with linearized pJL1, which generated TVY6 containing a complete replacement of the *PRC1* gene with the *HIS3* gene (Δ *prc1::HIS3*). TVY6 was separately transformed with the centromeric vector pSEYC58 containing the EcoRI to EcoRI fragment of the *PRC1* gene, and with pLV41 (Valls et al., 1987, 1990), which replaces glutamine 24 with lysine. TVY6 containing these two plasmids was used for all experiments with the misorting CPY mutant. Tom Stevens (University of Oregon, Eugene, OR) kindly provided both pLV41 and pJL1. PHY112 *MAT α* *ura3-52*; *his3- Δ 200*; *trp1- Δ 901*; *leu2-3,112*; *lys2-801*; *suc2- Δ 9*; *GAL*; Δ *vps15::HIS3* containing pPHY192 was used for all experiments with the *vps15* temperature-sensitive mutant. pPHY192 contains a glycine to alanine change at position 2 and a 30 amino acid COOH-terminal truncation in the *VPS15* structural gene (Herman et al., 1991). All endocytosis experiments used either GPY74-15C *MAT α* ; *ss1-3*; *leu2-3,112*; *ura3-52*; *trp1-289*; *his⁻*; or GPY385 (GPY74-15C Δ *pep4::LEU2*). Binding and internalization of ³⁵S- α -factor were performed with spheroplasts using previously published methods (Singer and Riezman, 1990).

Growth and Radiolabeling of Yeast Strains

Yeast strains were grown and converted to spheroplasts as described previously (Vida et al., 1990) except 0.2–0.5% glucuronidase (Dupont/New England Nuclear, Boston, MA) was included with lyticase during the first 15 min of digestion followed by a 10-min digestion with Novozyme (NOVO-Nordisk, Copenhagen, Denmark; used at 10 μ g/OD₆₀₀ U). Spheroplasts were pulsed with Tran³⁵S-label (ICN, Irvine, CA) for 2, 5, or 15 min and chased for varying times with 5 mM Met, 1 mM Cys, and 0.5% yeast extract depending on experimental needs. Spheroplasts were mock labeled under identical conditions for marker protein analysis.

Fractionation of Yeast Spheroplasts

Differential Extraction and Centrifugation. All spheroplast preparations were frozen over liquid nitrogen vapors as previously described (Vida et al., 1990), containing 25–30 OD₆₀₀ U/tube. After thawing, spheroplasts were washed on ice in the following scheme: 3–5 min with 250 mM sorbitol in 20 mM Hepes-KOH pH 6.8, 150 mM potassium acetate, and 5 mM magnesium acetate (transport buffer salts); 3 min with 50 mM sorbitol in transport buffer salts; 5 min with 2.0 M potassium acetate, 250 mM sorbitol, in transport buffer salts; and 5 min with 250 mM sorbitol in transport buffer salts. After each resuspension/incubation, the cells were harvested in a microfuge (model 235B, Fisher Scientific Co., Pittsburgh, PA) for 30–45 s at full speed (~13,000 g). The combined supernatants from the four washes were designated the S1 supernatant (cytosol) and resulting pellet designated the P1 (permeabilized cells). A small portion of the P1 pellet (usually 5–10% of the total) was used for in vitro assays reconstituting intercompartmental transport to the vacuole using 1 mg/ml exogenous cytosol as previously described (Vida et al., 1990). The remaining 90–95% of the P1 pellet was extracted three times with 1 ml of 0.8 M sorbitol, 10 mM triethanolamine-acetic acid, 1 mM EDTA, pH 7.6 (TEA buffer) by resuspension through a 26 gauge syringe needle (up and down 3–5 times). Every extraction was incubated on ice for 5 min and then the pellet was harvested in a microfuge for 5 min at 13,000 g. The combined supernatants from TEA buffer extraction were subjected to centrifugation in an SS-34 Sorvall rotor at 15,000 g

1. Abbreviations used in this paper: ALP, alkaline phosphatase; CPY, carboxypeptidase Y.

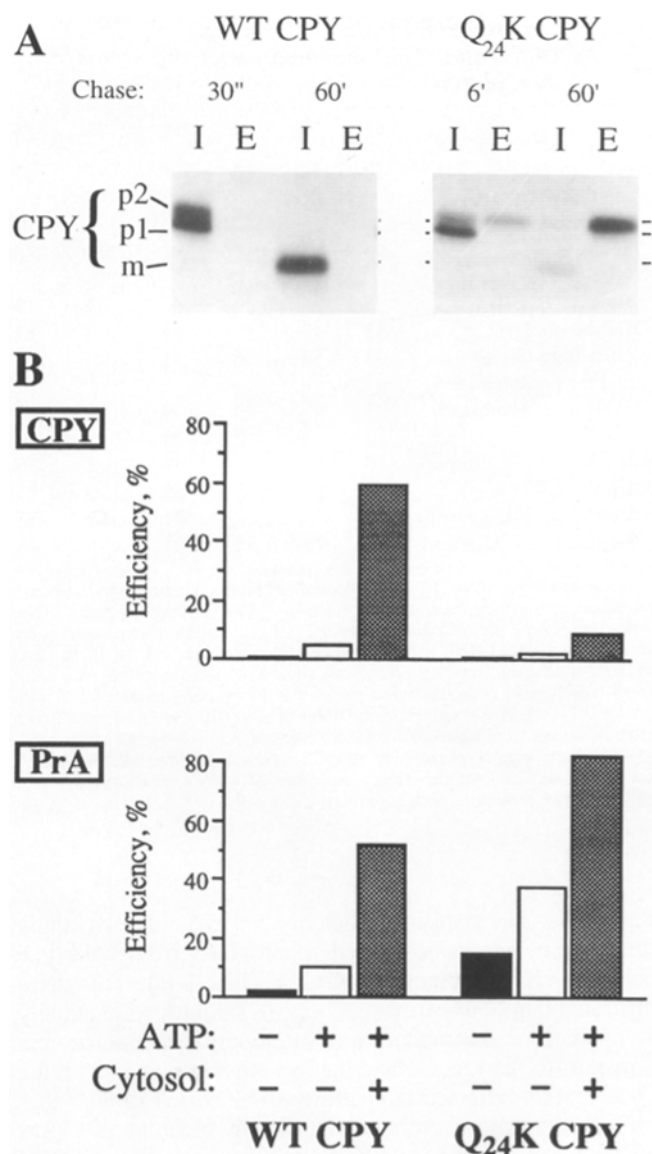


Figure 1. CPY with a mutant sorting signal is inefficiently transported in vitro. (A) In vivo. Spheroplasts from TVY6, containing plasmids encoding either the wild-type *PRC1* gene (WT CPY) or with glutamine 24 changed to lysine (Q₂₄K CPY), were pulsed with Tran³⁵S-label for 5 min and then chased for the indicated times. At each chase point, the cells were diluted into ice-cold isotonic buffer containing 10 mM NaN₃ and NaF and separated into an extracellular media (E) and intracellular cell pellet (I) fractions by centrifugation. Both fractions were then immunoprecipitated for CPY. (B) In vitro. Permeabilized cells were made from the 30 s (WT) and 6 min (Q₂₄K) chase points and incubated with buffer alone, ATP, an ATP regeneration system, and cytosolic protein extracts as indicated. CPY was immunoprecipitated first (*upper*) and then PrA was immunoprecipitated from the same samples (*lower*). Quantitation of efficiency is the quotient of the amount of mature protein and of Golgi-modified precursor plus mature expressed as a percent.

for 15 min. The resulting supernatant was designated the S2 and the small pellet was combined with large pellet in the microfuge tube and designated the pellet. The S2 supernatant was then subjected to centrifugation in a Beckman TLA100.1 rotor at 150,000 *g* (average) for 30 min and the resulting supernatant and pellet were designated the S3 and P3, respectively.

Equilibrium Sucrose Density Gradient Centrifugation. All sucrose solutions were made wt/wt, % with ultra-pure sucrose (J. T. Baker) from a 66% stock solution and contained 10 mM Hepes-KOH pH 7.6. The gradient consisted of 0.5 ml 61%, 1.0 ml 42%, 1.0 ml 37%, 1.5 ml 34%, 2.0 ml 32%, 2.0 ml 29%, 1.0 ml 27%, and 1.5 ml 22%. The P3 or P2 pellets were separately resuspended (or combined) in 1 ml TEA buffer with 0.8 M sorbitol and loaded on top of the gradient. The gradient was subjected to centrifugation in a Beckman SW41 rotor at 170,000 *g* for 16–20 h at 4°C. 16 0.72 ml fractions were collected from the top and any pellet resuspended in the last fraction. For radiolabeled samples, 300 μg of carrier BSA was added to each fraction and precipitated with TCA (10% final) for at least 30 min on ice. The TCA pellets were then processed and immunoprecipitated as described previously (Vida et al., 1990). All quantitation of gels was done with either a Molecular Dynamics Phosphorimager (Sunnyvale, CA) or a LKB densitometer (ULTRASCAN XL).

Enzyme Assays

All enzymes were measured using standard conditions as reported for NADPH cytochrome *c* reductase (Kubota et al., 1977), guanosine diphosphatase (Abeijon et al., 1989), Kex2p (Fuller et al., 1989). Total protein was measured with the BioRad Laboratories (Richmond, CA) protein dye binding assay according to the manufacturer's instructions.

Electron Microscopy

Approximately 250 OD₆₀₀ U of SEY6210 spheroplasts were subjected to differential extraction and centrifugation as described above. The P2 and P3 pellets were then separately applied to sucrose gradients and subjected to centrifugation for 18–20 h at 170,000 *g*. After taking 16 fractions from the top, fractions 1 and 2 (from the gradient of the P2 pellet) and fractions 5 and 6, and 12 and 13 (from the gradient of the P3 pellet) were pooled separately and 1 ml was immediately fixed with 1 ml of 3.0% glutaraldehyde, 1.6 M sorbitol, 10 mM Hepes-KOH, 2 mM EDTA, and 100 mM cacodylate buffer pH 7.5 for 1 h on ice. One ml of 0.1 M cacodylate pH 7.6 was then added and the fixed membranes were sedimented at 150,000 *g* (average) for 2 h at 4°C in a Beckman TLA100.1 rotor. The three pellets were then processed for electron microscopy as described (Hobman et al., 1992).

Results

CPY with a Mutant Sorting Signal Is Inefficiently Transported In Vitro

The reconstitution assay, measuring intercompartmental protein transport to the yeast vacuole, relies on radiolabeling spheroplasts to generate CPY precursors in vivo. The specificity of the assay for the Golgi-modified CPY precursor (p2) suggested that it reconstituted a single step in the vacuole delivery pathway (Vida et al., 1990). To investigate this specificity in more detail, a mutant form of CPY was used as a substrate for the in vitro assay. The 90 amino acid NH₂-terminal propeptide in CPY contains information that is both necessary and sufficient for directing this protein to the vacuole (Johnson et al., 1987; Valls et al., 1987). Indeed, a minimal sorting signal has been mapped to amino acids 24–27, QRPL. Normally, >95% of wild-type CPY is sorted to the vacuole. However, when glutamine 24 is replaced with lysine (Q₂₄K), 80–90% of newly synthesized CPY is not sorted to the vacuole but instead is secreted to the cell surface (Valls et al., 1990). After a 5-min pulse and 60-min chase, ~85% of the p2 precursor form of this mutant CPY was secreted from spheroplasts (Fig. 1, A). Importantly, the glutamine to lysine change in the propeptide does not affect proteolytic processing of the small amount of CPY that is delivered to the vacuole (Valls et al., 1990; and Fig. 1 A).

The transport properties of Q₂₄K CPY made it an excellent candidate for testing the specificity of the intercompartmental reconstitution assay. Using permeabilized sphero-

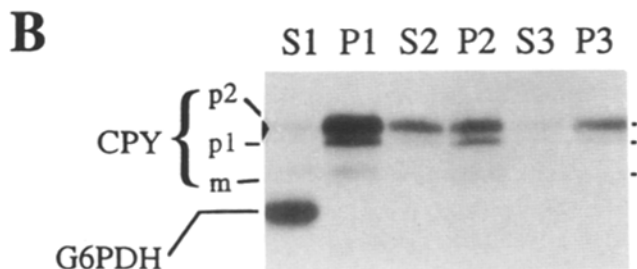
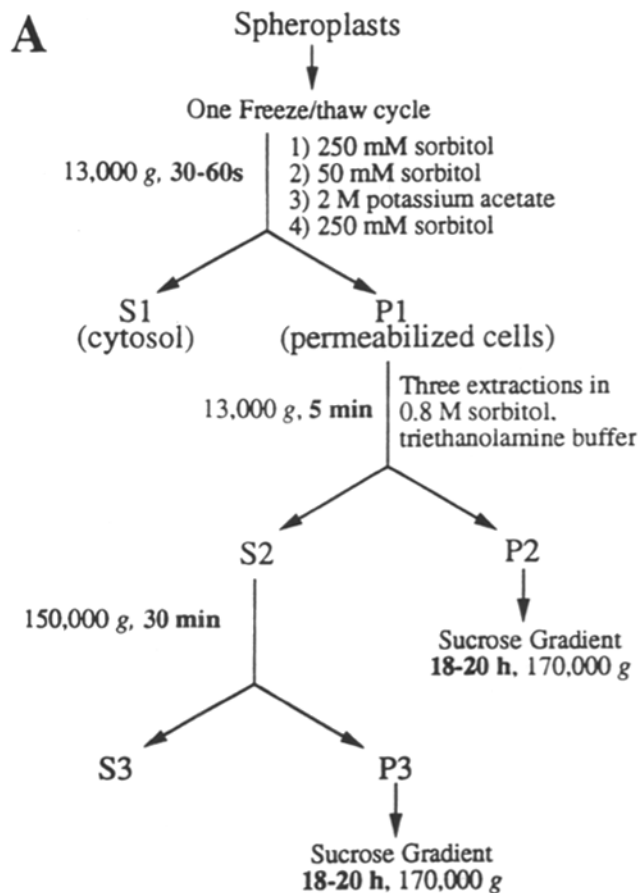


Figure 2. Differential extraction and centrifugation of permeabilized spheroplasts. (A) Flow chart depicting the standard subcellular fractionation protocol used in this study. (B) SEY6210 yeast spheroplasts were pulse labeled for 10 min and chased for 5 min at 30°C. The cells were extracted and subjected to differential centrifugation as described in Materials and Methods and depicted in A. Equivalent amounts of the various supernatant and pellet fractions were immunoprecipitated for CPY. The S1 supernatant and P1 pellet were coimmunoprecipitated for the cytosolic enzyme glucose-6-phosphate dehydrogenase (*G6PDH*).

plasts, nearly 60% of wild-type p2CPY is converted to mCPY after incubation with ATP and cytosol (Fig. 1 B). In contrast, only about 9% of Q₂₄K p2CPY was converted to mature CPY after a comparable incubation (Fig. 1 B). As an internal control, the *in vitro* transport characteristics of proteinase A (PrA) were examined with the same permeabi-

Table I. Distribution of Marker Proteins During Differential Centrifugation and Extraction of Yeast Spheroplasts

Marker	S1	P1	S2	P2	S3	P3
Total protein	82	18	12	6	7	5
Glucose-6-phosphate dehydrogenase (cytosol)	98	2	ND	ND	ND	ND
NADPH cytochrome <i>c</i> reductase (ER)	5	95	29	66	12	17
GDPase (Golgi)	53	47	35	12	<1	35
Kex2p (late Golgi)	43	57	36	21	<1	36
Alkaline phosphatase (vacuolar membrane)	<1	99	20	79	<1	19
p1CPY	7	93	20	73	<1	20
p2CPY	10	90	41	49	6	35
mCPY	60	40	5	35	5	<1
³⁵ S- α -factor 0°C	22	78	37	41	5	32
³⁵ S- α -factor 15°C	23	77	55	22	9	46

Spheroplasts from SEY6210 were prepared and separated into the indicated supernatant and pellet fractions according to Materials and Methods. Glucose-6-phosphate dehydrogenase, mCPY, and alkaline phosphatase were immunoprecipitated from radiolabeled cells that were pulsed for 15 min and chased for 45 min. The CPY precursors, p1 and p2, were immunoprecipitated from radiolabeled cells that were pulsed for 10 min and chased for 5 min. NADPH cytochrome *c* reductase, GDPase, and Kex2p were determined with enzyme assays from unlabeled cells. ³⁵S- α -factor was fractionated after binding the pheromone to spheroplasts at 0°C for 60 min and internalized at 15°C for 25 min with and without energy depletion. All values are expressed as the percentage of protein in each supernatant and pellet.

lized cells. The transport efficiency for PrA was essentially the same using permeabilized spheroplasts from wild-type cells or cells expressing Q₂₄K CPY (Fig. 1 B). This demonstrated that both permeabilized cell samples were equally competent for reconstitution of intercompartmental protein transport to the vacuole and the low efficiency seen with the Q₂₄K mutant reflected its inability to be sorted to the vacuole. The nearly sevenfold reduction in vacuolar transport efficiency of the mutant p2CPY relative to wild-type CPY suggested that sorting information in the CPY propeptide was required for efficient reconstitution of intercompartmental transport to the yeast vacuole. One explanation consistent with this result is that the intracellular sorting apparatus does not efficiently recognize Q₂₄K CPY and subsequent delivery to the vacuole or perhaps to another organelle that functions as the donor compartment is perturbed. To address this, subcellular fractionation techniques were used to identify organelles that contain the p2CPY precursor.

Fractionation of CPY Precursors by Differential Extraction and Centrifugation

Subcellular fractionation requires specific strategies to generate organelles for separation on the basis of their inherent characteristics, which can be followed by marker protein analysis. The radiolabeled precursors of CPY, particularly the Golgi-modified p2 precursor, were initially used as marker proteins to develop conditions for their separation. We chose to use permeabilized cells (rather than completely lysed cells) as the starting point in our fractionation studies for two reasons: (a) quantitative removal of cytosolic protein afforded a quick purification step and (b) with a functional as-

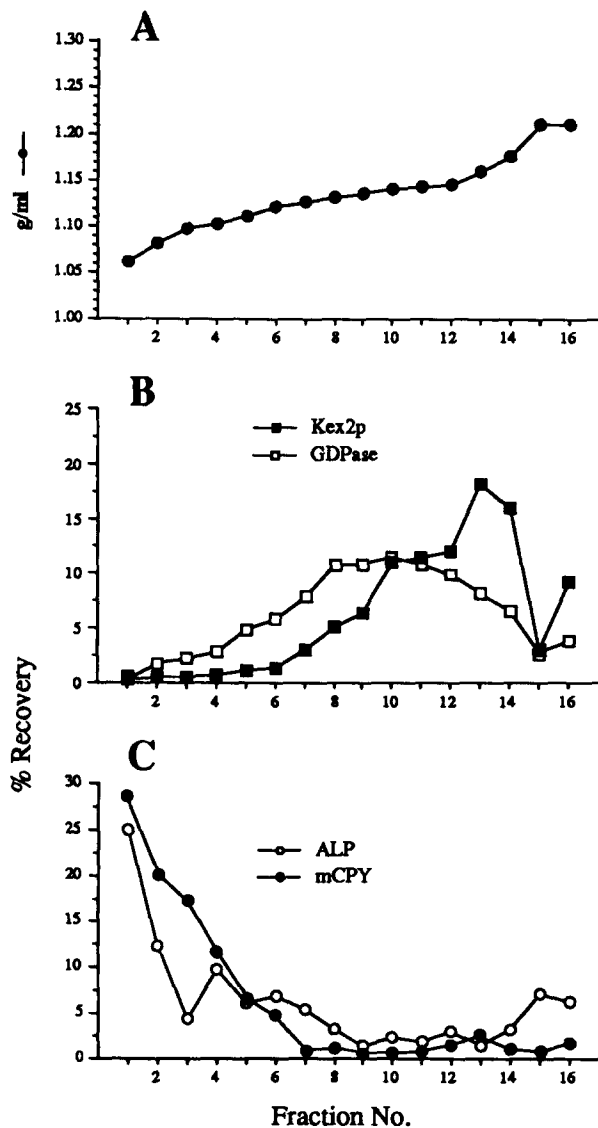


Figure 3. Equilibrium sucrose gradient fractionation of vacuolar and Golgi complex marker proteins. Yeast spheroplasts from SEY6210 were extracted and subjected to differential centrifugation as described in Materials and Methods. The 15,000 g P2 and 150,000 g P3 pellets were combined and subjected to equilibrium sucrose gradient centrifugation. (A) Density (g/ml) across a typical gradient. This was the shape of every gradient in this study. (B) The Golgi complex marker proteins GDPase and Kex2p were assayed as indicated. (C) The vacuolar marker proteins alkaline phosphatase and mCPY were immunoprecipitated from gradient fractions, as indicated, after pulse labeling cells for 15 min and chasing for 45 min.

say for intercompartmental transport to the vacuole, *in vitro* transport efficiency could be correlated with the fractionation behavior of p2CPY. We included a 2.0 M potassium acetate step during the washing protocol for permeabilized cell preparation (Fig. 2 A). This routinely ensured quantitative removal of cytosolic protein (S1 supernatant in Fig. 2 B and Table I). Organelles of the early secretory pathway (ER and Golgi complex) remained intact after these washes as measured by their ability to retain p1 and p2CPY (P1 pellet in Fig. 2 B and Table I). They also remained functional for

the intercompartmental *in vitro* protein transport assay (Fig. 1 B).

The buffers used to remove cytosol contain physiological levels of magnesium and potassium ions, which are required to maintain organelle associations with the permeabilized cells (Vida et al., 1990). Subsequent differential extraction with an isotonic, low ionic strength buffer (0.8 M sorbitol, 10 mM triethanolamine-EDTA) resulted in disassociation of permeabilized cells into dispersed organelles. After three such extractions, low speed centrifugation (15,000 g) separated precursor and mature forms of CPY (10' pulse, 5' chase) into the resulting supernatant (S2) and pellet (P2), respectively (Fig. 2 B and Table I). The amount of p2CPY that remained in the S2 supernatant fraction was variable depending on the precise pulse/chase conditions but was routinely at least 40–50% of the total (Table I). The p2CPY that did not sediment after repeated centrifugation at 15,000 g was not merely a result of lysed compartment(s), because it did sediment to the P3 pellet fraction after centrifugation at 150,000 g (Fig. 2 B and Table I). In contrast, >75% of the p1CPY precursor reproducibly sedimented in the P2 pellet fraction regardless of the pulse/chase conditions. Likewise, nearly 80% of the vacuolar marker protein alkaline phosphatase (ALP) sedimented to the P2 pellet fraction after centrifugation at 15,000 g. The sedimentation characteristics of resident organellar marker proteins were compared with radiolabeled CPY during differential extraction (Table I). As expected, p1CPY behaved like the ER marker NADPH cytochrome *c* reductase. In contrast, almost 50% of two Golgi marker proteins, Kex2p and GDPase, were in the S1 supernatant fraction whereas Golgi-modified p2CPY was recovered almost quantitatively in the P1 permeabilized cell pellet. Nearly half of the Kex2p activity in the S1 could be sedimented at 150,000 g, suggesting that a portion of this late Golgi complex marker was not solubilized during cell permeabilization (data not shown). Finally, when examined under the light microscope, the P2 and P3 pellets did not contain significant amounts of unbroken or permeabilized cells (data not shown).

A Large Portion of Golgi-Modified p2CPY Fractionates with a Novel Prevacuolar Compartment

The conditions for dissociating organelles from permeabilized cells led to the enrichment of certain organelles after differential centrifugation, which were further fractionated on sucrose density gradients. Under isopycnic conditions, this technique separates organelles on the basis of inherent buoyant density differences and when applied to yeast organelles, resolves the ER, mitochondria, plasma membrane, vacuole, and the Golgi complex (Walworth et al., 1989). We established conditions to optimize separation of the vacuole and the Golgi complex, such that their marker proteins banded near the top and the bottom of a sucrose density gradient, respectively. Essentially, this involved constructing a shallow density gradient of sucrose (Fig. 3 A). Two different vacuolar marker proteins, alkaline phosphatase (ALP) and mCPY, were recovered at the top of such a sucrose gradient (Fig. 3 C). Because ALP is an integral membrane protein and mCPY is a soluble luminal marker, the cofractionation of these two enzymes suggests that the vacuole remained intact during the different fractionation procedures. Although

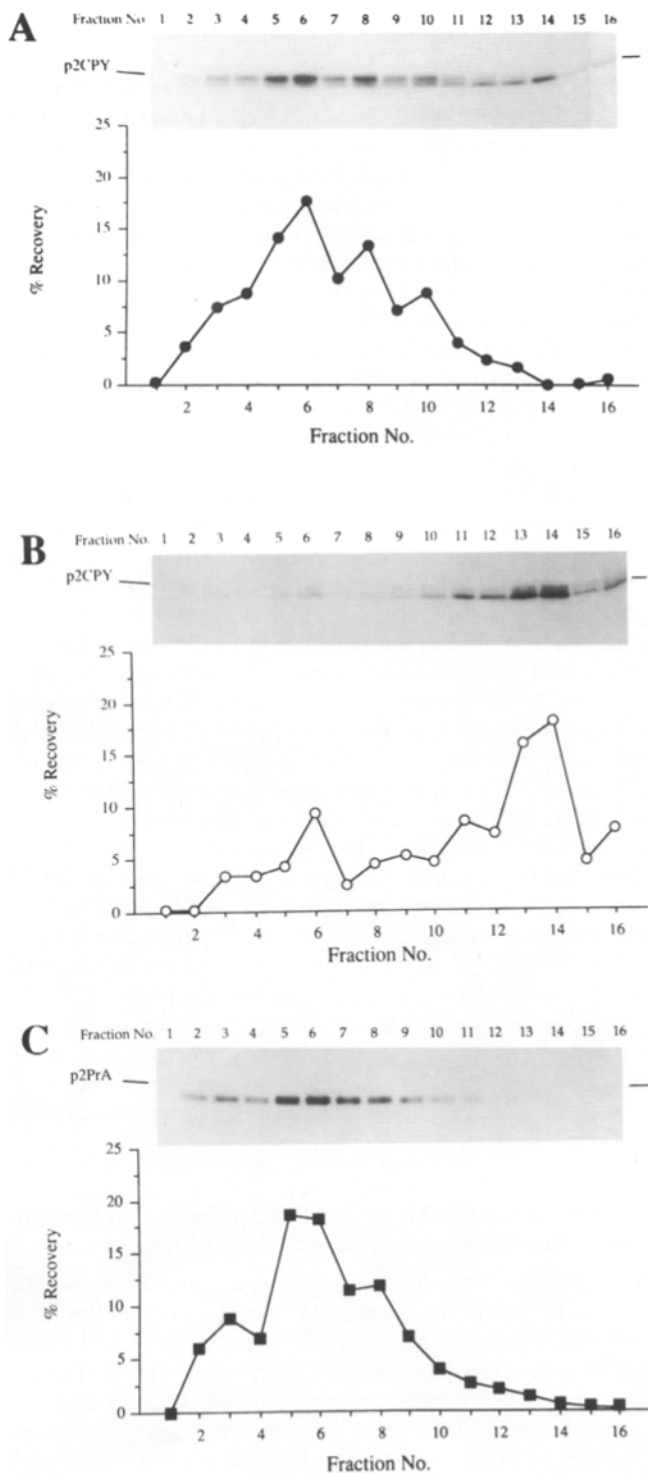


Figure 4. Equilibrium sucrose gradient fractionation of Golgi-modified vacuolar precursor proteins. The same TVY6 spheroplasts from Fig. 1 were extracted and subjected to differential centrifugation as described in Materials and Methods. The 150,000 g P3 pellet was subjected to equilibrium sucrose gradient centrifugation and immunoprecipitated for CPY and PrA. (A) Wild-type CPY (39,686,850 cpm loaded). (B) $Q_{24}K$ CPY, and (C) Wild-type PrA from the same gradient as $Q_{24}K$ CPY (33,903,350 cpm loaded).

the mCPY may have been released from ruptured vacuoles, this luminal marker also migrated to the top of the gradient after loading a resuspended membrane pellet at the bottom (data not shown). Under these conditions, both enzymes were recovered in a single peak at fraction 1 of the gradient, indicating that the vacuole had a low density of ≤ 1.059 g/ml (15%, wt/wt sucrose). In contrast to these vacuolar enzymes, two Golgi complex enzymes, GDPase and Kex2p, banded at higher density in the sucrose gradient at fractions 8–10 (1.133–1.140 g/ml) and fraction 13 (1.163 g/ml), respectively (Fig. 3 B).

The fractionation characteristics of Golgi-modified forms of CPY also were examined in this type of sucrose gradient. To enhance detection of these transient precursors, spheroplasts were radiolabeled using a 5-min pulse and a 30 s chase. The spheroplasts were subjected to differential extraction and centrifugation to generate a 150,000 g P3 pellet, which was further fractionated on the sucrose gradient. Immunoprecipitating each fraction with CPY antiserum showed that p2CPY migrated through the gradient with peak fractions at 6, 8, and 10 (Fig. 4 A). The p2CPY with peak fractions at 8 and 10 overlapped well with the Golgi complex markers, GDPase and Kex2p (Fig. 3 B). In contrast, the p2CPY at fraction 6, representing 35–40% of the total, was not coincident with the fractionation behavior of Golgi or vacuolar marker proteins (Fig. 3, B and C). This p2CPY at fraction 6 (Fig. 4 A) was less dense (1.119 g/ml) than GDPase at fraction 8 (Fig. 3 B) and was very well separated from the late Golgi-containing Kex2p at fraction 13 (Fig. 3 B). Because CPY is known to transit through this late Golgi sub-compartment (Graham and Emr, 1991), the low density peak of p2CPY was localized in a compartment distal or proximal to the Kex2p-containing late Golgi.

To determine if a kinetic relationship existed between the high and low density p2CPY-containing fractions, membranes from different times of chase were fractionated to distinguish which represented early and late compartments. After a 2-min pulse, cells were chased for 1 and 20 min to stop p2CPY at potentially different points in the secretory pathway. These time points were chosen because p2CPY represented 15–20% of the total radiolabeled CPY. At 1 min of chase, the majority of CPY was still present as the p1 precursor whereas at 20 min of chase the majority of CPY had been converted to the mature vacuolar form (data not shown). When membranes from these time points were fractionated on sucrose gradients, the amount of p2CPY that was recovered in fractions 3–7 increased over threefold after 20 min of chase, whereas recovery in fractions 9–13 decreased over threefold (Fig. 5). This demonstrated that early p2CPY moved from a compartment(s) with relatively high density, like the Golgi complex, to a compartment(s) with lower density, unlike both the late Golgi and the vacuole.

The $Q_{24}K$ Mutant Form of CPY Does Not Fractionate with the Low Density Prevacuolar Compartment

The fractionation profile of p2CPY on sucrose gradients suggested that its route to the vacuole involved transit through a low density compartment distal to the Kex2p-containing late Golgi compartment. The Kex2p-containing compartment appears to be the site where protein sorting occurs as it is the last compartment where vacuolar and secreted proteins are present together (Graham and Emr, 1991). To test

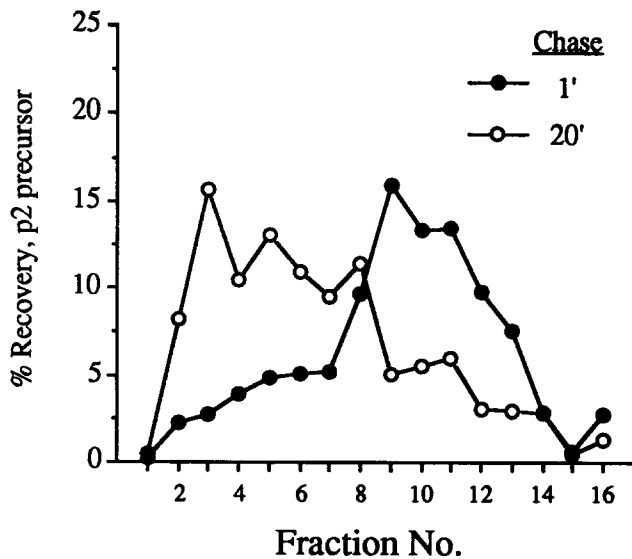


Figure 5. Golgi-modified CPY moves from dense to light compartments during pulse/chase analysis. Spheroplasts from SEY6210 were pulse labeled for 2 min and chased for 1 and 20 min at 30°C (as indicated). The cells were extracted and subjected to differential centrifugation as described in Materials and Methods. The 15,000 g P2 and 150,000 g P3 pellets were combined, subjected to equilibrium sucrose gradient centrifugation, and immunoprecipitated for CPY.

if the Q₂₄K mutant form of CPY transits through the low density compartment on its way to the cell surface, the mis-sorting mutant was fractionated on sucrose gradients using 150,000 g P3 pellets from the same cells shown in Fig. 1. Very little of Q₂₄K p2CPY was found in the gradient at

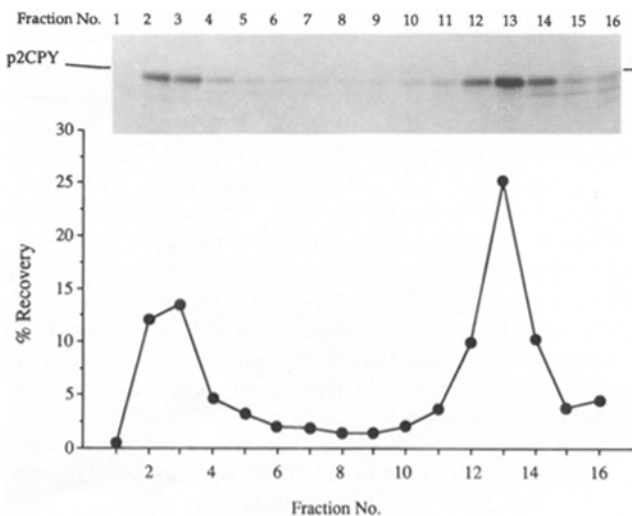


Figure 6. Equilibrium sucrose gradient fractionation of Golgi-modified CPY from a temperature sensitive *vps15* mutant blocked at the nonpermissive temperature. Spheroplasts from PHY112 harboring pPHY192 were shifted to 38°C for 2 min. The cells were then pulse labeled for 5 min and chased for 30 min. After the chase, the cells were extracted and subjected to differential centrifugation as described in Materials and Methods. The 15,000 g P2 and 150,000 g P3 membrane pellets were combined, subjected to equilibrium sucrose gradient centrifugation, and immunoprecipitated for CPY.

fraction 6 while the majority was found in fractions 12–14 (Fig. 4 B). This resulted in ~30-fold more Q₂₄K p2CPY in the denser region of the gradient compared to wild-type CPY (Fig. 4 A). In addition, fractions 12–14 also contained the late Golgi marker, Kex2p (Fig. 3 B). The Golgi-modified PrA precursor had a distinctly different fractionation pattern than Q₂₄K p2CPY within the same gradient. It behaved very similar to wild-type CPY (Fig. 4 C). From this analysis, a strong correlation existed between the fractionation of p2CPY or p2PrA in a compartment(s) of low density (Fig. 4) and the sorting capabilities of these precursors. Furthermore, this correlation extended to their efficiency for inter-compartmental transport *in vitro* (Fig. 1 B), suggesting that this low density compartment acted as a donor compartment.

A vps15 Temperature Sensitive Mutant Blocks Transport of Vacuolar Proenzymes in the Late Golgi Complex

In the previous experiments with wild-type cells, different times of chase were used to enrich for intermediate forms of vacuolar proteins in different organelles throughout the secretory pathway. Alternatively, temperature sensitive mutants can be used to block protein transport at distinct steps that lead to specific organelles quantitatively accumulating normally transient precursor proteins. Temperature-conditional *vps15* mutants that contain COOH-terminal truncations impose a reversible block in the vacuolar protein delivery pathway (Herman et al., 1991). Immediately upon shift to the nonpermissive temperature, these temperature sensitive mutants accumulate Golgi-modified p2CPY. To determine where in the pathway this block occurs, a temperature sensitive *vps15* mutant strain was shifted to the nonpermissive temperature (38°C) for 2 min, pulse labeled for 5 min, and chased for 30 min. Under these conditions, ~85% of the newly synthesized p2CPY accumulated intracellularly. After differential extraction and centrifugation, ~85% of the blocked p2CPY sedimented in the 15,000 g P2 pellet (data not shown). When the P2 and P3 pellets were combined and analyzed on a sucrose gradient, ~60% of the p2CPY was found in fractions 12–14 (Fig. 6). A smaller amount of p2CPY was observed at fractions 2–3 (Fig. 6). Unlike the p2CPY in fractions 12–14, the p2CPY in fractions 2–3 could not be sedimented at 100,000 g indicating that this CPY was released during centrifugation by lysis of the compartment. The 38°C labeling conditions used to impose the *vps15* block did not change the fractionation profile of Kex2p (data not shown). Thus, the peak of p2CPY at fraction 13 was identical to the fractionation pattern of Kex2p (Fig. 3 B), suggesting the transport block occurs in a late Golgi compartment.

Endocytosed α-Factor Cofractionates with the Low Density Prevacuolar Compartment

The organelle(s) in the low density region of the sucrose gradients (fractions 3–7) that contained substantial amounts of Golgi-modified vacuolar precursors did not cofractionate with known yeast marker proteins. Therefore, these fractions may have represented a unique compartment in the yeast cell, which could be a transport vesicle intermediate between the late Golgi and the vacuole. However, the density of this compartment was very similar, if not identical, to the density of the endosomal compartment in which internalized

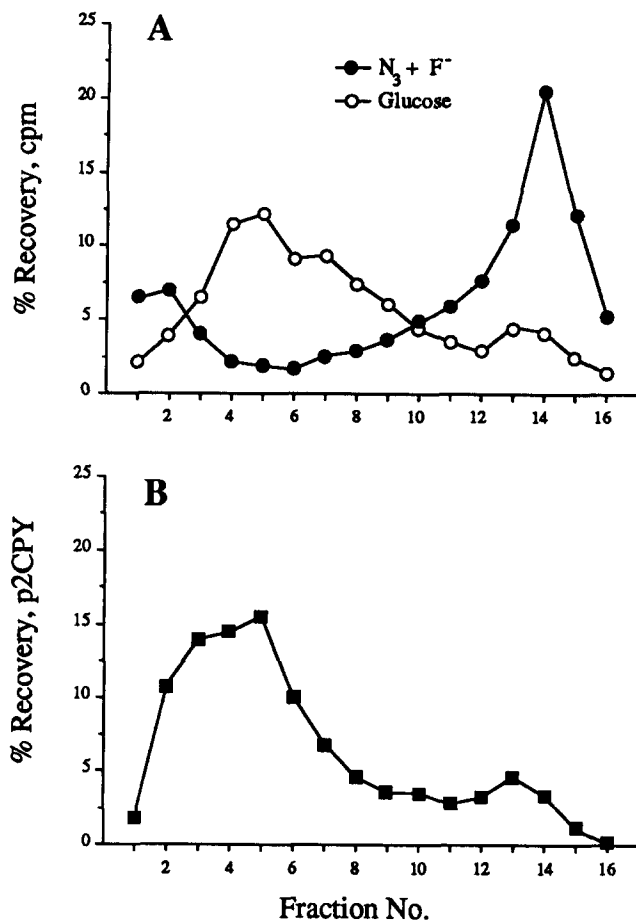


Figure 7. Equilibrium sucrose gradient fractionation of ^{35}S - α -factor internalized at 15°C . (A) Approximately 375,000 cpm of ^{35}S - α -factor were incubated with spheroplasts from GPY385 for 60 min at 0°C . The cells were briefly washed, and incubated at 15°C for 25 min in the presence or absence of NaN_3 and NaF as indicated. After internalization, the cells were extracted and subjected to differential centrifugation as described in Materials and Methods. The 150,000 g P3 pellet was subjected to equilibrium sucrose gradient centrifugation and the amount of radioactivity in each fraction was determined with a scintillation counter. The starting ^{35}S - α -factor was judged to be $>98\%$ pure when analyzed with both SDS-PAGE and TLC. (B) Spheroplasts from GPY74-15C were pulse labeled at 30°C for 5 min and chased for 1 min. The cells were washed briefly, then incubated at 0°C for 60 min, and at 15°C for 25 min. After this incubation scheme, the radiolabeled CPY comprised $\sim 20\%$ p1CPY, 65% p2CPY, and 15% mCPY. The cells were extracted and subjected to differential centrifugation as described in Materials and Methods. The 150,000 g P3 membrane pellet was subjected to equilibrium sucrose gradient centrifugation and each fraction was immunoprecipitated for CPY.

α -factor has been detected during endocytosis and transport to the vacuole (Singer and Riezman, 1990).

To examine this apparent similarity, α -factor was fractionated under the above conditions after receptor-mediated internalization. Spheroplasts were incubated with ^{35}S - α -fac-

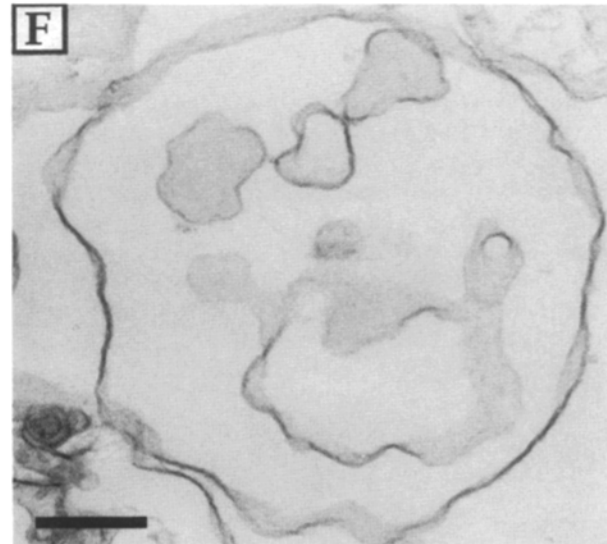
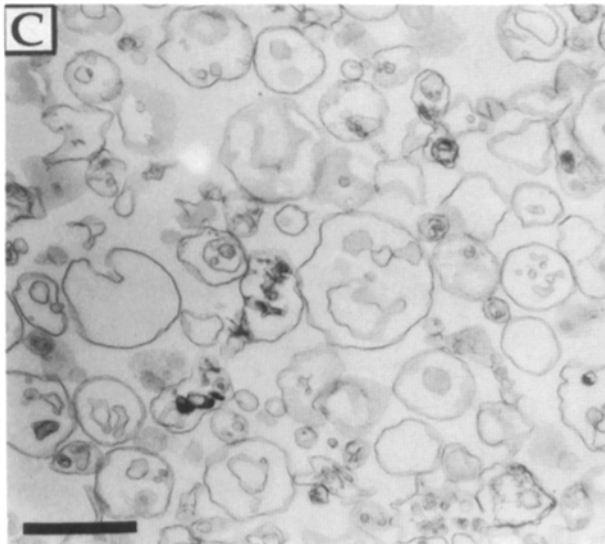
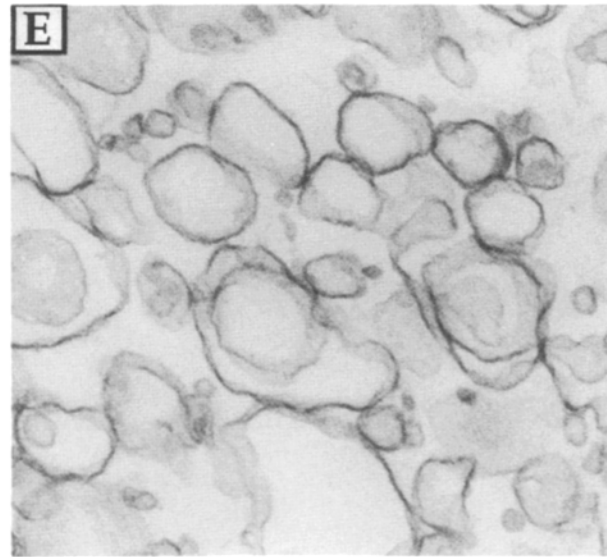
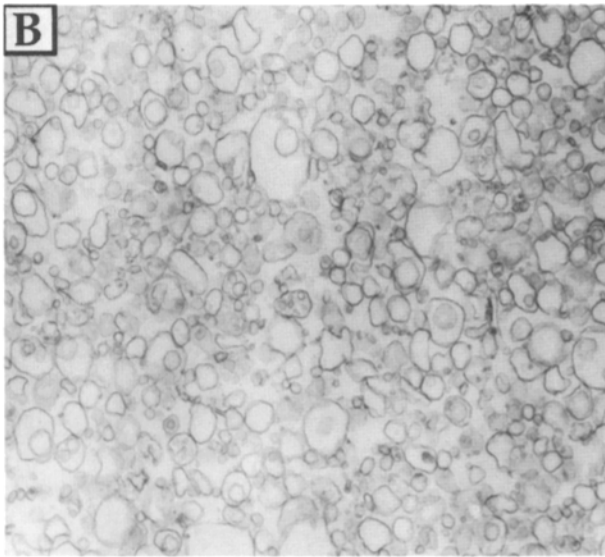
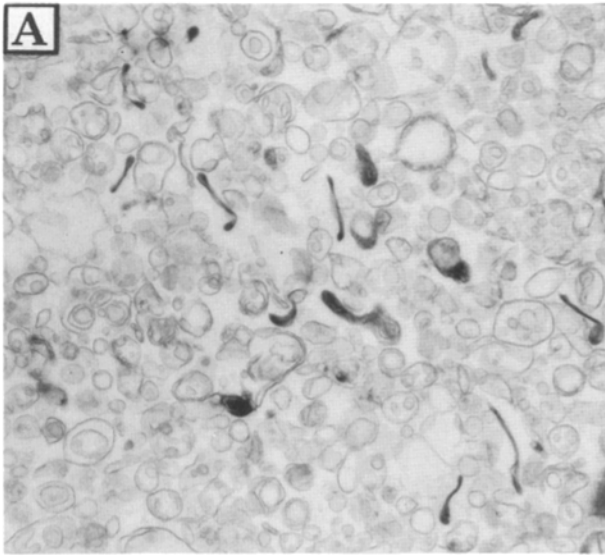
tor at 0°C for 60 min, resulting in 50–60% of the ligand bound to the cell surface. Internalization was performed at 15°C to slow membrane transport, which allows the internalized ligand to localize between the vacuole and the plasma membrane (Singer and Riezman, 1990). A parallel incubation was performed in the presence of energy poisons (NaN_3 and NaF), which blocks internalization and traps the ligand at the plasma membrane. During differential centrifugation, nearly 80% of the ligand remained associated with the P1 permeabilized cell pellet (Table I). After centrifugation to equilibrium on sucrose gradients, ^{35}S - α -factor exhibited an energy dependent difference in its banding profile. Under conditions that block internalization (NaN_3 and NaF), a sharp peak of ^{35}S - α -factor at fraction 14 was recovered in the dense region of the gradient (Fig. 7 A), consistent with the density of the plasma membrane (Singer and Riezman, 1990). In the absence of energy poisons, the amount of radiolabeled α -factor at fraction 14 dropped \sim threefold and resulted in a lower density peak between fractions 4–6 with a broad shoulder between fractions 7–10 (Fig. 7 A). When p2CPY was examined under these conditions, namely incubation of cells at 15°C , it cofractionated with the low density peak of internalized ^{35}S - α -factor between fractions 4–6 (Fig. 7 B), which suggested this compartment was endosome-like rather than a transport vesicle intermediate.

Morphology and Polypeptide Composition of Membrane Pellets Enriched for the Golgi Complex, the Prevacuolar Compartment, and the Vacuole

Various sucrose density gradient fractions were examined by electron microscopy to determine their morphological characteristics. Three pairs of fractions from two sucrose gradients were pooled, fixed, and sedimented to obtain three membrane pellets: fractions 1 and 2 from a gradient loaded with a 15,000 g P2 pellet; and fractions 5 and 6, and 12 and 13 from a gradient loaded with a 150,000 g P3 pellet. The general appearance of all these pellets consisted of membrane-enclosed compartments of varying size (Fig. 8). The Golgi complex pellet (fractions 12 and 13 from P3) consisted of relatively small 150–250 nm vesicles that contained numerous electron-dense 300–1,000 nm long tubule cisternae (Fig. 8, A and D). This structure is reminiscent of both mammalian (Mellman and Simons, 1992) and yeast Golgi (Preuss et al., 1992). The vacuole-enriched pellet (fractions 1 and 2 from P2) contained large 750–1,250 nm membrane-bounded organelles, some of which contained multiple smaller 50–200 nm vesicles within them (Fig. 8, C and F). The endosomal-enriched pellet (fractions 5 and 6 from P3) was devoid of Golgi-like tubule cisternae and contained membrane-enclosed compartments of 250–400 nm in diameter (Fig. 8, B and E). Thus, the morphology of the endosome-enriched membrane fraction was distinct from both the vacuole and the Golgi complex, suggesting that it may have consisted of a unique membrane-enclosed compartment(s).

The protein composition of these membrane pellets was

Figure 8. Morphology of membranes enriched for the vacuole, Golgi complex, and the prevacuolar, endosomal compartment. Approximately 2.5×10^9 cells from SEY6210 were converted to spheroplasts and subjected to differential extraction and centrifugation as described in Materials and Methods. The P2 and P3 membrane pellets were then subjected to centrifugation on sucrose gradients. Fractions 12 and 13 enriched for Golgi complex (A, D) and fractions 5 and 6 enriched for the prevacuolar, endosomal compartment (B, E) were



taken from the gradient loaded with the 150,000 g P3 membrane pellet, and fractions 1 and 2 enriched for the vacuole (C, F) were taken from the gradient loaded with the 15,000 g P2 membrane pellet. These were fixed for 60 min on ice and sedimented at 150,000 g for 2 h. The pellets were then processed for electron microscopy. Bars: (A-C) 1 μm ; (D-F) 0.262 μm .

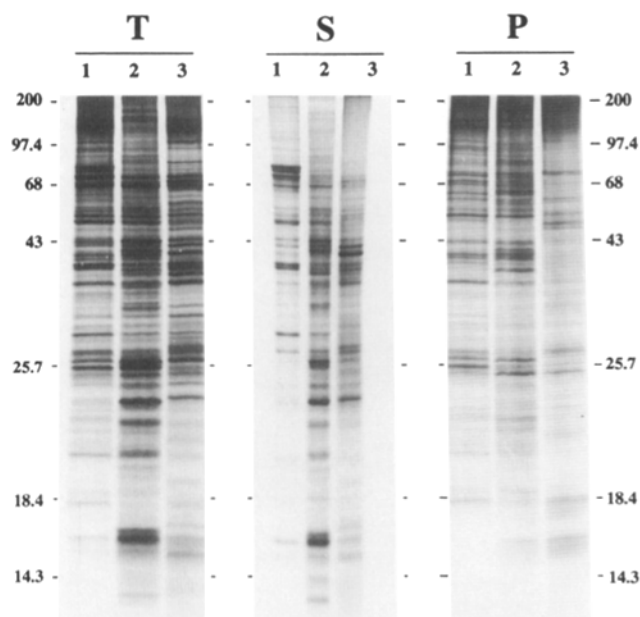


Figure 9. Polypeptide composition of membranes enriched for the vacuole, Golgi complex, and the prevacuolar, endosomal compartment. Approximately 2.5×10^9 cells from SEY6210 were converted to spheroplasts and subjected to differential extraction and centrifugation as described in Materials and Methods. The P2 and P3 pellets were then subjected to centrifugation on sucrose gradients. Fractions 12 and 13 enriched for Golgi complex (lane 1) and fractions 5 and 6 enriched for the prevacuolar, endosomal compartment (lane 2) were taken from the gradient loaded with the 150,000 g P3 pellet, and fractions 1 and 2 enriched for the vacuole (lane 3) were taken from the gradient loaded with the 15,000 g P2 pellet. The total protein content (T) and that which was soluble (S) or sedimentable (P) in sodium carbonate were analyzed from each fraction on a 12% SDS polyacrylamide gel. 5 μ g of protein were loaded in each lane and visualized with a silver stain. Molecular weights are given in kilodaltons.

compared using one-dimensional SDS-PAGE, which further indicated their differences. Even in total protein, the polypeptide composition differed significantly between the fractions enriched for the late Golgi complex (Fig. 9 lane 1, T), the low density endosome-like compartment (Fig. 9 lane 2, T), and the vacuole (Fig. 9, lane 3, T). Detection of these differences was enhanced after extracting the membranes with sodium carbonate into soluble and sedimentable fractions. The soluble portion (peripheral and luminal proteins) from each consisted of about 10 major polypeptides most of which were unique (Fig. 9, lanes 1-3, S). The polypeptide composition from the sodium carbonate pellets (10-16 different proteins) was also unique among these fractions with the most conspicuous difference observed in the 25-26 kD range (Fig. 9, lanes 1-3, P). Together with the morphology of these membranes (Fig. 8), the polypeptide composition suggested that the low density endosome-like compartment was distinct from both the late Golgi and the vacuole.

Discussion

The vectorial movement of newly-synthesized yeast vacuolar proteins, like CPY, through the early secretory pathway was

initially characterized in the *sec* mutants (Stevens et al., 1982). Those studies provided the first definitive evidence that CPY went from the ER to the Golgi complex before delivery to the vacuole. The yeast Golgi complex, although morphologically elusive, is composed of functional subcompartments like the mammalian organelle (Bowser et al., 1991; Cunningham et al., 1989; Franzusoff and Schekman, 1989). Specific carbohydrate modification, temperature sensitive transport blocks, and better marker proteins have allowed functional dissection of the yeast Golgi complex. For example, the early Golgi *sec7* block (Franzusoff and Schekman, 1989), acquisition of α 1,3-linked mannose residues, and transit through the Kex2p-containing late Golgi (Graham and Emr, 1991), all occur in separate Golgi subcompartments. The identification of a distinct fractionation pattern for p2CPY in this study might have suggested the existence of yet another Golgi subcompartment distal to the Kex2p-containing compartment. However, the evidence supports models that have predicted the presence of an endosome-like organelle between the Golgi and the vacuole (Seeger and Payne, 1992; Raymond et al., 1992).

Clarification of the vacuolar protein delivery pathway was possible through the use of yeast mutants, a mutant form of CPY, subcellular fractionation techniques, and a specific intercompartmental reconstitution assay. From the results obtained in this analysis, the model for localization of vacuolar proteins, like CPY, is shown in Fig. 10. This model builds upon the previous demonstration that p2CPY transits through the Kex2p-containing late Golgi compartment and confirms this as the site for sorting vacuolar precursors away from proteins destined for secretion (Graham and Emr, 1991). However, for the first time a new compartment distal to the late Golgi is demonstrated to exist in the yeast vacuolar protein delivery pathway. After p2CPY travels through the late Golgi, the precursor localizes to a post-Golgi, prevacuolar compartment rather than being sorted directly to the vacuole. Proteolytic maturation of p2CPY occurs after delivery from the prevacuolar compartment to the vacuole. The prevacuolar compartment is most likely an endosome because endocytosed α -factor appears to colocalize with this organelle after internalization at 15°C, conditions that slow transport from the cell surface to the vacuole. This suggests that endocytic and biosynthetic protein traffic to the yeast vacuole converge at a common compartment, analogous to the late endosome in mammalian cells (Griffiths et al., 1988).

This refined model for protein transport to the vacuole rests on several lines of evidence. (a) The physical separation of organelle(s) that contain p2CPY from both the late-Golgi marker, Kex2p, and the vacuole markers alkaline phosphatase and mCPY indicates the prevacuolar compartment is distinct. Equilibrium sucrose gradient analysis establishes the p2CPY-containing prevacuolar compartment to have a density of ~ 1.12 g/ml, the late Golgi ~ 1.16 g/ml, and the vacuole no more than 1.06 g/ml. (b) Pulse/chase analysis demonstrated a kinetic relationship between the Golgi complex and the prevacuolar compartment. At early chase times, p2CPY fractionated with the higher density late Golgi Kex2p compartment but at later chase points p2CPY fractionated with the lower density prevacuolar compartment. This places the order of the p2CPY-containing low density prevacuolar compartment after the late-Golgi but before the

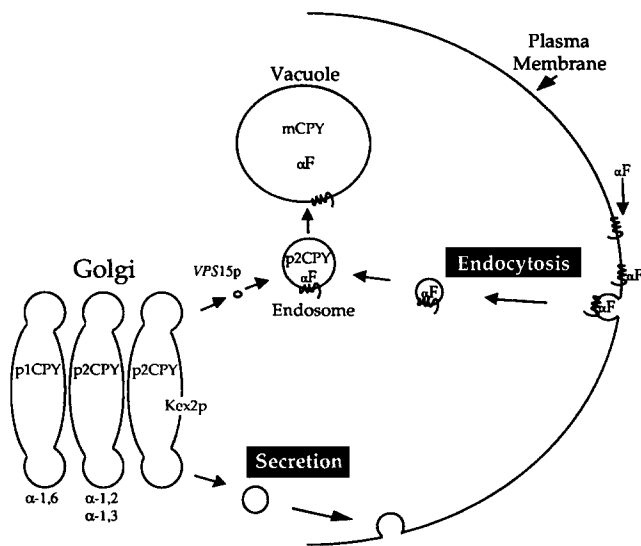


Figure 10. Compartmental model for protein delivery to the vacuole through biosynthetic and endocytic routes. The mating pheromone alpha-factor (α F) is shown undergoing receptor-mediated endocytosis and a degradation in the vacuole after transport from an endosome. The Golgi-modified p2CPY precursor is shown undergoing the sorting step in the late Golgi before transport to an endosome followed by maturation in the vacuole. See Discussion for other details.

vacuole. (c) α -factor fractionated with the same density as the prevacuolar compartment after internalization at 15°C, which suggests that this organelle is endosome-like rather than a transport vesicle intermediate between the late Golgi and the vacuole. (d) Reconstitution analysis demonstrated a strong correlation between highly efficient vacuolar delivery of p2CPY *in vitro* and its localization to the low density prevacuolar compartment *in vivo*. (e) Sorting determinants in the propeptide of CPY are required to localize p2CPY in the low density prevacuolar compartment. The amount of Golgi-modified CPY from the Q₂₄K missorting mutant was significantly decreased (three- to fourfold) in this intermediate compartment. However, the mutant CPY cofractionated with the late-Golgi Kex2p marker. (f) Electron microscopy revealed that the prevacuolar organelle fraction was composed of 250–400 nm compartments distinct from both the larger vacuole and the smaller and tubule-like Golgi compartments. This data also corroborates that this prevacuolar compartment is most likely not a transport vesicle by virtue of its relatively large, nonuniform size. (g) Polypeptide composition is another independent level of criteria to establish the prevacuolar compartment as being distinct from both the Golgi and the vacuole.

The subcellular fractionation results presented in this study independently confirm earlier work that implicates the Kex2p-containing late Golgi as the sorting site for soluble vacuolar proteins from the default pathway of secretion (Graham and Emr, 1991). Compelling evidence was obtained with analysis of two different components that affect sorting (a) a point mutation in the propeptide sorting signal of CPY and (b) the *vps15* temperature-sensitive vacuolar protein sorting mutant. In both cases, Golgi-modified p2CPY cofractionates with the Kex2p-containing late Golgi indicating

that events within this compartment promote proper protein sorting to the vacuole away from the secretory pathway. Interestingly, introduction of a positive charge in the CPY propeptide (Q₂₄K) not only results in substantial missorting to the cell surface, but also a kinetic delay in transport through the late Golgi complex. Together these observations indicate that the Kex2p-containing late Golgi compartment functions as the yeast cell equivalent of the mammalian TGN (Griffiths and Simons, 1986; Mellman and Simons, 1992). With this in mind, the *vps15* temperature sensitive transport block provides the first demonstration for a cytoplasmic factor, the VPS15 protein kinase, that functions in the regulation of protein sorting in the late Golgi complex.

Several points about the kinetics of vacuolar delivery in yeast become clear from our analysis of sorting and transport. The data suggest that the rate limiting event in p2CPY delivery to the vacuole is not in movement from the α 1,3 mannosyltransferase to the Kex2p compartment or from the Kex2p compartment to the endosome but rather in transport from the endosome to the vacuole. Sucrose gradient fractionation of p2CPY from different chase points shows that cofractionation with the late Golgi marker, Kex2p, is very transient. Over multiple time points only small amounts of wild-type p2CPY or p2PrA were observed to cofractionate with the Kex2p-containing late Golgi (fractions 12 and 13; Vida and Emr, unpublished results). This suggests that the kinetics of p2CPY movement from the dense Kex2p-containing late Golgi compartment to the less dense prevacuolar compartment are relatively fast. In contrast, a large pool of p2CPY cofractionates with the prevacuolar endosome compartment at several chase points. Thus, the slowest step in vacuolar delivery of CPY is most likely not movement from the late Golgi to the endosome, but rather transport from the endosome to the vacuole. Importantly, p2CPY was not observed to cofractionate with vacuolar marker proteins, indicating that proteolytic maturation is not rate limiting.

Techniques to resolve yeast organelles on sucrose gradients are fast-becoming the standard method to characterize the subcellular location of an unknown protein (Walworth et al., 1989; McCaffrey et al., 1991; Bowser et al., 1991; Newman et al., 1992). The fractionation behavior of newly-synthesized p2CPY or p2PrA delineate the compartments involved with vacuolar delivery of these precursor proteins. For CPY, the p1 form generally follows the fractionation behavior of the ER while the p2 form fractionates with the Golgi complex and the endosome-like prevacuolar compartment. No stable resident marker proteins for this low density compartment exist yet, only the transient passage of Golgi-modified vacuolar precursors and internalized α -factor through this compartment permit its detection. The steady state amount of both these transient markers within this compartment is negligible. Future efforts will be directed toward the identification of integral membrane proteins associated with the prevacuolar endosomal compartment to serve as functional markers like the recently identified 57-kD protein of the mammalian prelysosomal compartment (Park et al., 1991). Interestingly, the density of this organelle in yeast (\sim 1.12 g/ml) is nearly identical to the density of the mammalian prelysosomal compartment (Mullock et al., 1989; Park et al., 1991).

The molecular mechanism for protein transport from the prelysosome to the lysosome is poorly understood. One ma-

question is whether transport carrier vesicles shuttle cargo between these organelles or if the late endosome "matures" into a lysosome. The genetic and biochemical approaches in yeast should provide insight into this problem. The reconstitution assay for measuring intercompartmental transport to the vacuole in yeast is most efficient for the step between the prevacuolar intermediate and the vacuole. The characteristics of this assay imply that a carrier vesicle functions to transfer contents to the vacuole (Vida et al., 1990). To demonstrate this unequivocally will require isolation of these vesicles separated from the donor compartment, the endosome, and the target organelle, the vacuole. Additionally, purification of the endosome-like compartment will enable efforts to identify resident marker proteins and could permit reconstitution of vacuolar protein delivery into a cell-free donor/acceptor reaction.

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