Parallel Secretory Pathways to the Cell Surface in Yeast

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Abstract. *Saccharomyces cerevisiae* mutants that have a post-Golgi block in the exocytic pathway accumulate 100-nm vesicles carrying secretory enzymes as well as plasma membrane and cell-wall components. We have separated the vesicle markers into two groups by equilibrium isodensity centrifugation. The major population of vesicles contains Bgl2p, an endoglucanase destined to be a cell-wall component, as well as Pma1p, the major plasma membrane ATPase. In addition, Snc1p, a synaptobrevin homologue, copurifies with these vesicles. Another vesicle population contains the periplasmic enzymes invertase and acid phosphatase. Both vesicle populations also contain exoglucanase activity; the major exoglucanase normally secreted from the cell, encoded by *EXG1*, is carried in the population containing periplasmic enzymes. Electron microscopy shows that both vesicle groups have an average diameter of 100 nm. The late secretory mutants *sec1*, *sec4*, and *sec6* accumulate both vesicle populations, while neither is detected in wild-type cells, early *sec* mutants, or a *sec13 sec6* double mutant. Moreover, a block in endocytosis does not prevent the accumulation of either vesicle species in an *end4 sec6* double mutant, further indicating that both populations are of exocytic origin. The accumulation of two populations of late secretory vesicles indicates the existence of two parallel routes from the Golgi to the plasma membrane.

As is the case for other microorganisms with a rigid cell wall, the driving force for growth and morphogenesis in *Saccharomyces cerevisiae* is turgor pressure. The cell wall prevents growth except at locations where it is modified and allowed to expand (Harold, 1990). Turgor pressure is uniform over the entire cell surface, and a newborn cell initially grows isotropically. However, upon initiation of a bud, growth becomes localized to the bud tip. Once a bud reaches a certain size, isotropic expansion again dominates to complete formation of the daughter cell (Farkas et al., 1974; Lew and Reed, 1993). In both polarized and isotropic growth, surface expansion is accomplished by the delivery and fusion of secretory vesicles carrying plasma membrane and cell-wall components; mutants specifically blocked in secretion are defective in cell-surface growth (Novick and Schekman, 1979, 1983; Novick et al., 1980; Pryer et al., 1992). Secretory vesicles also transport wall-modifying enzymes such as glucanases, which are thought to soften the wall to allow expansion and insertion of new wall materials (Matile et al., 1971; Hien and Fleet, 1983). Therefore, it is the direction of secretion that localizes growth and determines the shape of the cell (Harold, 1990; Lew and Reed, 1993).

There is strong indication that actin organization is responsible for establishing the directionality of secretion (Lew and Reed, 1993; Bretscher et al., 1994). Cortical actin patches are clustered at sites of new cell-wall synthesis throughout the cell cycle (Adams and Pringle, 1984; Kilmartin and Adams, 1984), and an aberrant actin cytoskeleton results in delocalized growth and the accumulation of secretory vesicles (Novick and Botstein, 1985; Johnston et al., 1991; Liu and Bretscher, 1992). Secretory vesicles may be transported along actin cables, and actin may be responsible for localizing the ER and Golgi complex. The latter possibility is consistent with a model for fungal morphogenesis suggested by Bartnicki-Garcia et al. (1989), in which the location of a "vesicle supply center" affects the polarity of secretion. This situation is supported by immunoelectron microscopy, which shows a clustering of Golgi at areas of cell-wall synthesis (Preuss et al., 1992).

Besides the cytoskeleton, recognition molecules on the vesicle and plasma membranes determine the location of vesicle fusion (Ferro-Novick and Jahn, 1994; Rothman, 1994). Candidates for such membrane markers at nerve terminals are syntaxin (t-SNARE) on the plasma membrane and synaptobrevin (v-SNARE) on synaptic vesicles. According to the "SNARE hypothesis," t-SNAREs on the target membrane interact with v-SNAREs on transport vesicles to establish the selectivity of vesicle docking before fusion. Specific SNAREs are thought to regulate fusion at each trafficking step, including ER to Golgi and Golgi to other compartments (Söllner et al., 1993; Rothman, 1994; Søgaard et al., 1994). The yeast SNAREs involved in Golgi complex to plasma membrane transport are Sso1p and Sso2p, which are syntaxin homologues (Aalto et al., 1993), and Snc1p and Snc2p, which are ho-

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mologues of synaptobrevin (Protopopov et al., 1993). It is not clear whether SNAREs simply identify membranes for the maintenance of organelle compartmentation, or if they also determine the spacial location of fusion events.

Members of the Rab family of small GTP-binding proteins are also believed to have a role in the regulation of membrane fusion (Ferro-Novick and Novick, 1993). While the precise role of Rabs is unclear, they are suspected to have a proofreading function in stabilizing the interaction between t-SNAREs and v-SNAREs (Novick and Brennwald, 1993; Søgaard et al., 1994). Like SNAREs, specific Rabs are believed to act at each stage of membrane trafficking. The first Rab protein discovered is the yeast Sec4p, which regulates the fusion of vesicles with the plasma membrane (Salminen and Novick, 1987).

Along with many other genes essential for secretion, the gene for Sec4p was identified in a screen for conditionally lethal yeast mutants blocked at various stages of protein transport (Novick et al., 1980). Depending on the stage in the pathway at which the block occurs, these *sec* mutants accumulate specific secretory organelles as well as glycoproteins when shifted to restrictive temperature (Novick and Schekman, 1979; Novick et al., 1980). Analysis of double *sec* mutants confirmed the order of transport in the secretory pathway from ER to Golgi to cell surface (Novick et al., 1981). The combination of genetic and biochemical techniques in yeast has contributed greatly to the elucidation of molecular mechanisms involved in secretion (Pryer et al., 1992; Rothman, 1994).

As in yeast, secretion in many cell types is a polarized process. Polarized epithelial cells provide an example in which different populations of Golgi-derived vesicles transport proteins to separate domains of the plasma membrane (Rindler et al., 1984; Mostov et al., 1992). The basolateral and apical surfaces of these cells have distinct protein compositions. Specialized secretory cells, such as some endocrine cells and nerve cells, have both a constitutive and regulated secretory pathway and offer another case where different species of post-Golgi vesicles are targeted to the cell surface (Gumbiner and Kelly, 1982).

Yeast secretion is thought to be a rapid, constitutive process, and wild-type cells have a relatively low abundance of secretory vesicles (Novick and Schekman, 1979). However, temperature-sensitive *sec* mutants enable purification of functional vesicles that accumulate at restrictive temperature (Holcomb et al., 1987; Walworth and Novick, 1987). Previous purification and characterization of post-Golgi secretory vesicles, as well as immunoelectron microscopy, has indicated one population of vesicles transporting plasma membrane components and secreted enzymes (Brada and Schekman, 1988; Holcomb et al., 1988). We now show that these cargoes are transported by at least two vesicle populations that accumulate in *sec* mutants blocked in Golgi to cell-surface transport.

Materials and Methods

Strains, Media, and Materials

Reagents for media were obtained from Difco Laboratories, Inc. (Detroit, MI). Minimal media contained 0.67% yeast nitrogen base, 2% glucose, and required amino acids. Phosphate-depleted rich medium contained 1% bacto-yeast extract, 2% bacto-peptone, and 4% glucose, and was depleted of phosphate as described by Rubin (1973). Yeast strains used in this work are listed in Table I. Standard genetic techniques were used for strain construction (Guthrie and Fink, 1991). The lithium acetate procedure (Schiestl and Gietz, 1989) was used for yeast transformations. The 2μ plasmid pDB31 carries the *SUC2* gene fused with the triosephosphate isomerase promoter (Brada and Schekman, 1988).

Nycodenz, Sephacryl S-1000, protease inhibitors, and reagents for enzyme assays were obtained from Sigma Chemical Co. (St. Louis, MO), except SDS for the guanosine diphosphatase assay was from Bio-Rad Laboratories (Hercules, CA) (for low phosphate contamination). Zymolyase-100T was from ICN Biochemicals, Inc. (Irvine, CA). Endo-β-*N*-acetylglucosaminidase (Endo H)[1] was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Electron microscopy reagents were from Polysciences, Inc. (Warrington, PA). All other materials were of reagent-grade purity. Optical density and absorbance values were read on a spectrophotometer (DU-50; Beckman Instruments, Inc., Fullerton, CA).

Purification of Secretory Vesicles

Cells were grown at 25°C to early exponential phase (OD_{600} of 0.7) in 1–2 liters minimal medium, harvested by centrifugation, and resuspended in phosphate-depleted rich medium. Growth was continued at 25°C for 90 min to induce acid phosphatase secretion. Cells were then shifted to 37°C for 2 h, harvested, and washed in ice-cold 10 mM NaN_3. After a 15-min incubation on ice in 0.1 M Tris-H_2SO_4 (pH 9.4), 50 mM β-mercaptoethanol, 10 mM NaN_3, cells were washed in 1.4 M sorbitol, 50 mM KPi, pH 7.5, 10 mM NaN_3 (spheroplast wash buffer). Cells were then resuspended in spheroplast wash buffer containing 0.15 mg/ml Zymolyase-100T (at least 12 ml buffer/g wet-packed cells; 1 liter produces ~2.5 g cells), and incubated at 37°C for 45 min with gentle agitation. After harvesting at 2,000 *g*, spheroplasts were washed twice gently in ice-cold spheroplast wash buffer.

1. *Abbreviations used in this paper:* Endo H, endo-β-*N*-acetylglucosaminidase H; TEA, triethanolamine-EDTA-acetic acid.

Table I. Yeast Strains Used in This Study

Strain	Genotype*	Source
NY10	*MATa ura3-52*	P. Novick, Yale University, New Haven, CT
NY13	*MATa ura3-52*	P. Novick
NY17	*MATa sec6-4 ura3-52*	P. Novick
NY414	*MATa sec13-1 ura3-52*	P. Novick
NY431	*MATa sec18-1 ura3-52*	P. Novick
ABY228	*MATa sec13-1 ura3-52*	NY10 × NY414
ABY227	*MATa sec6-4 sec13-1 ura3-52*	ABY228 × NY17
ABY701	*MATa sec6-4 ura3-52 [TPI::SUC2]*	transformed NY17
ABY702	*MATa ura3-52 [TPI::SUC2]*	transformed NY13
DBY1829	*MATa his3-Δ200 leu2-3,122 lys2-801 trp1-1 ura3-52*	T. Huffaker, Cornell University, Ithaca, NY
ABY704	*MATa sec6 trp1-1 leu2-3,122 ura3-52*	DBY1829 × NY17
RH268-1C	*MATa end4 leu2 his4 ura3 bar1-1*	H. Riezman, Biozentrum University, Basel, Basel, Switzerland
ABY717	*MATa end4 sec6-4 trp1-1 leu2 ura3*	ABY704 × RH268-1C

*Genes borne by plasmids are indicated by brackets.

Spheroplast pellets were placed on ice for 10 min between washes to allow the pellets to loosen, which were gently resuspended using a plastic transfer pipette to minimize lysis. All remaining steps were carried out at 4°C or on ice. Washed spheroplasts were resuspended in lysis buffer (15 ml/g wet-packed unspheroplasted cells) which contained 0.8 M sorbitol with 10 mM triethanolamine, 1 mM EDTA, adjusted to pH 7.2 with acetic acid (TEA), and protease inhibitors (30 mM PMSF, 1 mM benzamidine, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin). Cells were transferred to a glass Dounce homogenizer (Wheaton Industries, Millville, NJ) and lysed with 20 strokes of the A pestle (Walworth and Novick, 1987). A 700-*g* spin for 10 min in a rotor (SS34; Sorvall Instruments Division, DuPont Co., Newton, CT) generated the P1 (pellet) and S1 (supernatant) fractions. The S1 fraction was spun at 13,000 *g* for 20 min in the same rotor to generate P2 (large contaminating organelles) and S2 (secretory vesicles and soluble proteins). The S2 fraction was then divided into 17-ml tubes and centrifuged for 1 h at 100,000 g_{av} in a rotor (SW28.1; Beckman Instruments, Inc.). Membrane pellets (P3) were overlaid with 300 μl of 0.8 M sorbitol/TEA and placed on ice for 2–3 h to allow easy resuspension with a P-1000 Pipetman (Rainin Instrument Co., Woburn, MA). The protein concentration in the supernatant (S3) was used to normalize experiments. This correlated well with the wet-packed weight of unspheroplasted cells (50 mg ± 5.5 mg protein in S3/g cells, in three *sec6* and two wild-type experiments). All data shown correspond to 250 mg protein in the S3 soluble protein fraction (~5 g cells).

For gradient fractionation, a 16-ml 15–30% Nycodenz, 0.8-M sorbitol/TEA continuous gradient was formed with a gradient maker attached to a fractionator (Auto Densi-Flow II C; Haakebuchler Instruments, Inc., Saddle Brook, NJ). The P3 membranes were adjusted to 35% Nycodenz in a total volume of 1–1.5 ml and loaded into the bottom of the formed gradient using an 8.9-cm needle (5184; Becton Dickinson and Co., Mountain View, CA). Gradients were centrifuged in a Beckman SW28.1 rotor at 100,000 g_{av} for 19 h, and 0.6-ml fractions were collected from the top using an Auto Densi-Flow II C fractionator. Fraction densities were determined by reading refractive indices on a refractometer (Bausch & Lomb Inc., Instruments & Systems Division, Rochester, NY) and converting these values to grams per milliliter based on a standard curve generated by five weighed standards.

For gel filtration fractionation, the P3 membranes were resuspended in 3 ml 0.8 M sorbitol/TEA and loaded onto a 150 × 1.5 cm Sephacryl S-1000 column (Sigma Chemical Co.). The column was eluted with 0.8 M sorbitol/TEA at a flow rate of ~8 ml/h, and 5-ml fractions were collected.

Enzyme Assays

Acid phosphatase was assayed by the method of Van Rijn et al. (1972). Samples were diluted up to fourfold, and 60-μl aliquots were added to 0.2 ml *p*-nitrophenyl phosphate (11 μmol/ml), 0.1 M sodium acetate (pH 3.8), and 0.1% Triton X-100. Tubes were incubated 60 min at 30°C, and reactions were terminated by adding 2 ml of 0.4 M NaOH. Absorbance was determined at 415 nm. Units are expressed as micromoles *p*-nitrophenol liberated per minute.

Exoglucanase activity was determined basically as described by Santos et al. (1979). The reaction buffer contained 0.25% *p*-nitrophenyl-β-D-glucopyranoside as substrate, 50 mM acetate buffer, pH 5.5, 10 mM NaN_3, and 0.1% Triton X-100. 20-μl aliquots were added to 250 μl reaction buffer, and incubation was performed at 37°C for 3–6 h (reactions were linear for at least 6 h). Samples were boiled for 3 min to terminate the reaction and centrifuged at 2,000 *g* for 5 min in an Eppendorf microcentrifuge to remove precipitated protein. 2 ml of 4% Na_2CO_3 was added to 0.2 ml supernatant, and absorbance was read at 415 nm. Units are expressed as micromoles *p*-nitrophenol liberated per hour.

ATPase activity was determined essentially as described by Bowman and Slayman (1979). This procedure is similar to others used to assay for the Mg^{2+}-dependent, vanadate-sensitive plasma membrane ATPase (Tschopp and Schekman, 1983; Chang and Slayman, 1991). A 0.5-ml reaction mixture contained 10–20 μl fraction sample, 5 mM Na_2ATP, 5 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 25 μg pyruvate kinase, 5 mM NaN_3, and 10 mM Pipes, adjusted to pH 6 with Tris. Reactions were incubated at 30°C and terminated after 60–90 min by adding 0.5 ml 10% TCA. Inorganic phosphate was quantified according to Fiske and Subbarow (1925). Activity is expressed as arbitrary units based on absorption at 820 nm.

For determining invertase activity, fractions were diluted 10–200-fold and assayed by the method of Goldstein and Lampen (1975), except 0.1% Triton X-100 was included in the enzyme reaction buffer. Units are expressed as micromoles glucose produced per minute.

GDPase assays were performed basically as described (Abeijon et al., 1989; Yanagisawa et al., 1990). Fraction samples were diluted fivefold and 5 μl was added to 100 μl reaction buffer (containing 20 mM imidazole-HCl, pH 7.4, 2 mM $CaCl_2$, 0.1% Triton X-100, and 9 mM GDP or CDP as control for nucleotide specificity) and incubated at 30°C for 15 min. Reactions were stopped by adding 150 μl of 2% SDS, and inorganic phosphate was determined by a modified Fiske-Subbarow method (Ames, 1966). Activity is expressed as arbitrary units based on absorption at 820 nm.

Electron Microscopy

For negative staining, fraction samples were fixed for 1 h on ice in 1% glutaraldehyde, 3% fresh formaldehyde, 0.8 M sorbitol, and 40 mM KPi, pH 7. 20 μl samples were applied to Formvar/carbon-coated copper grids and incubated at room temperature for 15 min. Grids were stained with 2% phosphotungstic acid, pH 7.2, as described (Cluett et al., 1993), except staining was for 3 × 5 s. Grids were air dried, and examined and photographed in an electron microscope (301; Phillips Electronic Instruments Co., Mahwah, NJ) at 80 kV.

For thin sections, membranes were fixed as above and pelleted at 100,000 *g* for 1 h in a rotor (TLA 100.3; Beckman Instruments, Inc.). Pellets were washed in 40 mM KPi and postfixed in 0.5% OsO_4/0.8% $Fe_3(CN)_6$, 40 mM KPi, pH 7.2, for 1 h on ice. Samples were washed in H_2O and stained en bloc in 0.5% aqueous uranyl acetate overnight at 4°C, dehydrated in graded ethanol, and embedded in Spurr's resin. A similar method was used to prepare whole cells. Embedded membrane pellets were split in half and reembedded so that thin sections could be cut across the depth of the pellet. Sections were stained with uranyl acetate and lead citrate, and photographed in a Phillips 301 electron microscope at 60 kV.

Other Procedures

Protein concentration was determined using a protein assay (Bio-Rad Laboratories) based on the dye-binding technique of Bradford (1976).

SDS-PAGE was performed according to Laemmli (1970). 60-μl aliquots of each fraction were diluted with sample buffer (15 μl, with 10% SDS, 5% β-mercaptoethanol, and 25 mM EGTA) and heated at 100°C for 5 min, except samples for the Pma1p and Vph1p Westerns and Con A overlay were diluted 100-fold and heated at 37°C for 15 min. 5-μl samples were loaded for gels stained with silver (Wray et al., 1981) and 10-μl samples were loaded for blots. For silver-stained gels, molecular weights were estimated using myosin, phosphorylase B, BSA, carbonic anhydrase, soybean trypsin inhibitor, and aprotinin as standards. For Western blots, proteins were transferred to nitrocellulose (0.1 μm pore size; Schleicher & Schuell Inc., Keene, NH) using a semidry blotter (Integrated Separation Systems, Natick, MA). Blots were blocked with 5% milk in TBS-Tween 20, pH 7.5, for 1 h and incubated with primary antibodies for 1–2 h: for Pma1p, 1:20,000 dilution of polyclonal antiserum No. 838 (Serrano et al., 1991); for Snc1p, 1:2,000 dilution of polyclonal antiserum No. 3177 (Søgaard et al., 1994); for Bgl2p, 1:10,000 dilution of polyclonal antiserum No. 4 (Mrša et al., 1993); for Exg1p, 1:4,000 dilution of polyclonal antiserum (Ramírez et al., 1989); for Kar2p, 1:50,000 dilution of polyclonal antiserum (Rose et al., 1989). After probing with HRP-conjugated goat anti-rabbit secondary antibody, the blots were developed using an enhanced chemiluminescence kit (Amersham Corp.). For the Con A overlay, blots were blocked with 2% gelatin in TBS-Tween 20, pH 6.9, incubated for 1 h in 0.1 μg/ml HRP-conjugated Con A in TBS-Tween 20, pH 6.9, and developed using enhanced chemiluminescence. Colored molecular weight markers (Sigma Chemical Co.) were used for determination of molecular weights on blots.

For deglycosylation of proteins, 2 μl of Endo H (1 mU/μl) was added to a reaction mixture containing 30 μl gradient fraction sample, 10 mM NaN_3, 30 mM PMSF, 1 mM benzamidine, 0.1 M acetate buffer, pH 5.5, and 0.1% Triton X-100. Control reactions were the same except without the addition of Endo H. After incubating for 18 h at 37°C, samples were diluted in Laemmli sample buffer, boiled, and subjected to SDS-PAGE and Western blot analysis as described above.

Results

Two Populations of Membranes Accumulate in the *sec6-4* Mutant

Electron microscopy has shown that the late secretory mutant *sec6-4* accumulates a large number of 100-nm vesicles

(Novick et al., 1980). Vesicles from this mutant have been isolated following the periplasmic enzyme invertase as a marker (Walworth and Novick, 1987). We used the same basic method to spheroplast and lyse cells and to fractionate organelles by differential centrifugation (Fig. 1). A major difference in our procedure was that rather than shifting cells to low (0.2%) glucose at restrictive temperature to derepress invertase secretion, cells were shifted to high (4%) glucose, phosphate-depleted media to induce the secretion of acid phosphatase, another periplasmic enzyme marker for secretory vesicles. A high level of glucose also favors the production or activity of other proteins transported by vesicles, including plasma membrane ATPase (Serrano, 1983; Chang and Slayman, 1991; Eraso and Portillo, 1994). To allow detection of invertase in the same preparation, cells carried a 2μ plasmid containing the *SUC2* gene (encoding invertase) driven by the non-glucose repressible triosephosphate isomerase promoter (Brada and Schekman, 1988). After a 2-h shift to the restrictive temperature (37°C), cells were spheroplasted in 1.4 M sorbitol and lysed in 0.8 M sorbitol. The sorbitol concentration of 0.8 M was maintained throughout the remainder of our purification procedure to preserve the integrity of organelles (Makarow, 1985; Walworth and Novick, 1987). The lysate was subjected to differential centrifugation: a low-speed spin (700 *g*) to remove unlysed cells and large debris (P1), and a 13,000-*g* spin to pellet most of the ER, nuclei, mitochondria, vacuole, plasma membrane, and Golgi (P2) (Walworth et al., 1989; McCaffrey et al., 1991). A high-speed spin (100,000 *g*) harvested most of the remaining membranes (P3), which were gently resuspended and loaded at the bottom of a continuous Nycodenz gradient. Fractions were collected from the top after centrifugation of membranous structures to equilibrium. Only soluble proteins remained at the bottom of the gradient.

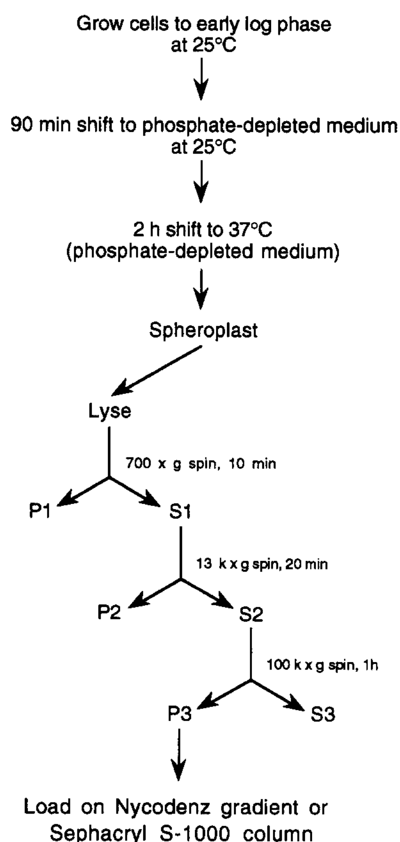

Figure 1. Purification of vesicles from late *sec* mutants.

As shown in Fig. 2, gradient fractionation of membranes from *sec6-4* cells resulted in two peaks of enzyme activities that are absent from fractionation of wild-type cells. One peak contains ATPase and exo-β-1,3-glucanase activities, while a higher density peak contains the periplasmic enzymes invertase and acid phosphatase, as well as another peak of exo-β-1,3-glucanase activity. A smaller peak of ATPase activity was also consistently found in the higher density peak, in agreement with earlier work showing that the same vesicle species can transport periplasmic enzymes and Pma1p, the major plasma membrane ATPase (Brada and Schekman, 1988; Holcomb et al., 1988). In addition, Western blots (Fig. 3 *A*) showed that the endo-β-1,3-glucanase Bgl2p (not detected by the assay for exoglucanase), which is a cell-wall component (Mrša et al., 1993), and the synaptobrevin homologue Snc1p also cofractionate with the less dense enzyme peak, while the exoglucanase encoded by *EXG1* (Nebreda et al., 1986; Ramírez et al., 1989) cofractionates with the higher density peak.

Western blots also indicated that Pma1p peaks with the lower density membranes, confirming that the detected ATPase activity is due to this protein (Fig. 3 *A*). The multiple bands detected are likely to be degradation products of the ATPase, also seen in other reports (Brada and Schekman, 1988; Kuchler et al., 1993). Caution is needed when detecting Pma1p by Western blot, as the protein aggregates and does not run into the gel at high concentrations even when samples are not boiled. It was necessary, therefore, to first determine by Western blot analysis the optimal dilution for detection of Pma1p (Fig. 3 *C*). Very little Pma1p is detected in equivalent dilutions of fractions from the wild-type gradient (Fig. 3 *B*). The detected ATPase activity is Mg^{2+}-dependent, insensitive to azide, and sensitive to low concentrations of vanadate, with no activity detected in 40 μM vanadate (data not shown), consistent with the characteristics of the major plasma membrane ATPase activity (Willsky, 1979; Serrano, 1988). While both vacuoles and mitochondria also contain a [H^+] ATPase, the mitochondrial ATPase is inhibited by azide, and the vacuolar enzyme is insensitive to vanadate (Uchida et al., 1988).

As only two yeast exoglucanases are detected in culture supernatants, both encoded by *EXG1* (Nebreda et al., 1986, 1987; Ramírez et al., 1990), we considered whether the exoglucanase activity detected in both vesicle peaks is a product of this gene. Exg1p has been shown to have two very different glycosylation states: one form contains 11% carbohydrate, while a highly glycosylated and heterogeneous form has ~40% carbohydrate content (Nebreda et al., 1987; Ramírez et al., 1989). The relative amounts of the two forms vary depending on growth conditions (Hien and Fleet, 1983). Since the lower density peak contains a slightly greater exoglucanase activity but apparently does not contain a peak of Exg1p, we reasoned that the activity detected in these membranes is due either to a different enzyme or to the highly glycosylated form of Exg1p that is

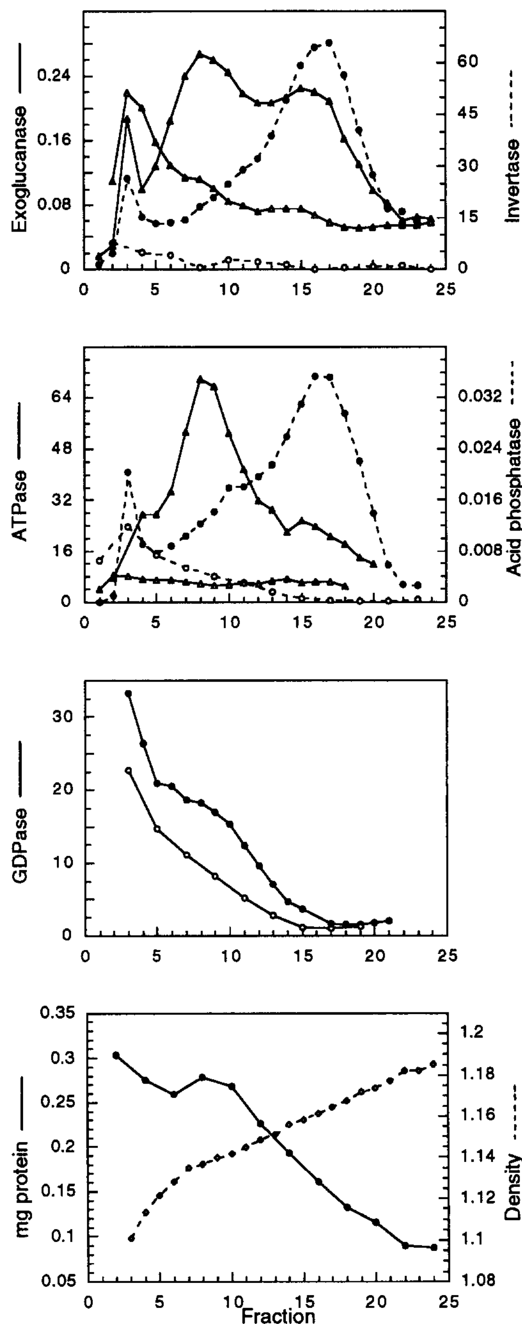

Figure 2. Enzyme activities in Nycodenz gradient fractions. *sec6-4* (*closed symbols*) or wild-type cells (*open symbols*) transformed with pDB31 were grown and fractionated as shown in Fig. 1. The P3 fraction was adjusted to 35% Nycodenz and loaded (1.5 ml) into the bottom of a 16 ml 15–30% Nycodenz gradient in 0.8 M sorbitol. After centrifugation to equilibrium, fractions (600 μl) were collected from the top and assayed for enzyme activity, protein and density. Activities are units per fraction. The separation of two enzyme peaks in *sec6* fractions, absent from wild-type fractions, indicates the accumulation of two membrane populations with distinct densities in the *sec6* mutant.

not easily detected by Western blot (Ramírez et al., 1990). To differentiate between the two possibilities, we treated samples from each peak fraction with Endo H to remove N-linked carbohydrate (Fig. 3 *D*). Because the highly glycosylated form has a lower specific activity than the less glycosylated form (Ramírez et al., 1989), deglycosylation should reveal more Exg1p in the lower density peak if the activity in both peaks is due to this protein. Western blot indicates a slightly more intense Exg1p band in the Endo H–treated samples of both peaks, suggesting the presence of the highly glycosylated form of the enzyme; however, there is clearly much more deglycosylated protein in the higher density peak. Therefore, the two peaks of exoglucanase activity likely result from two different proteins rather than from the sorting of the same protein into different pathways.

The Bgl2p-containing membranes peak at a density of 1.14 g/ml (Fig. 2) and appear to be the major population of accumulated membrane as indicated by SDS-PAGE of gradient fractions (Fig. 4), while the periplasmic enzyme–containing membranes peak at a density of 1.165 g/ml and seem to be a minor population of accumulated membrane, as there is no clear corresponding protein peak on the SDS gel. Analysis of total protein in gradient fractions (Fig. 2) further indicates that the lower density membranes are more abundant. The SDS gel of *sec6* gradient fractions also shows that the major proteins cofractionating with the lower density peak have apparent molecular masses of 76, 29, 27, and 19 kD; these proteins are not detected in wild-type fractions (Fig. 4).

The densities of the two accumulated membrane populations, as well as the enzyme profiles of the gradients, were consistent in all experiments in which cells were grown under the conditions described above, except the relative amount of exoglucanase activity in the two peaks varied slightly. Additionally, while results are shown for cells shifted to restrictive temperature for 2 h, similar results were obtained for cells shifted for 1 or 3 h, except the activities in both peaks increased with longer shifts (not shown). A result similar to wild-type fractionation was obtained for *sec6-4* cells grown at permissive temperature (25°C, not shown).

The apparent low abundance of the periplasmic enzyme–containing membranes may be due in part to the presence of highly glycosylated proteins that may be difficult to detect or would not run as clear bands on gels. However, Con A blot overlay of fraction samples indicates a much greater abundance of glycoproteins in the less-dense membrane peak (Fig. 5). Alternatively, a portion of the higher density vesicles may be lost during fractionation. However, as shown in Fig. 6 *A*, 42% of the acid phosphatase activity originally present in the low-speed supernatant (S2) was recovered in the high-speed pellet (P3), which represents 31% of the activity present in total lysed cells (S1). The remaining soluble activity (S3) likely derives from enzyme released from contaminating cell walls and disrupted organelles. The yield for invertase in these preparations was somewhat lower (Fig. 6 *B*); however, if cells did not carry the 2μ *SUC2* plasmid but were instead derepressed for invertase secretion by shifting into low (0.2%) glucose medium at restrictive temperature, the yield for invertase was similar to that obtained for acid phosphatase. Additionally, up to 90% of the acid phosphatase activity loaded into the Nycodenz gradient (P3) was present in the periplasmic enzyme–containing membrane peak. Similar yields of periplasmic enzyme were ob-

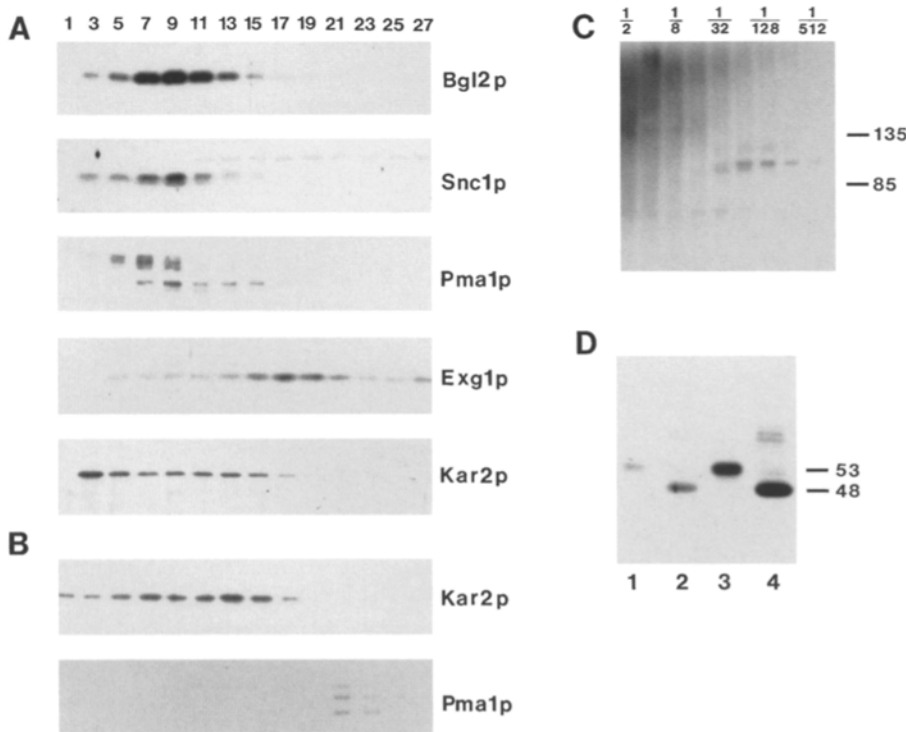

Figure 3. Western blots showing distribution of secretory vesicle and ER markers in Nycodenz gradient fractions from (*A*) *sec6* and (*B*) wild type. Cells were fractionated as described in Figs. 1 and 2. Odd-numbered fractions (as indicated at the top) were resolved by SDS-PAGE and transferred to nitrocellulose for immunodecoration with polyclonal antisera against the proteins indicated. (*C*) Determination of optimal fraction dilution for the Pma1p Western blot. Twofold serial dilutions of fraction 10 from the *sec6* gradient were heated in sample buffer at 37° for 15 min and resolved by SDS-PAGE, then transferred for Western blotting with antiserum against Pma1p. Because the ATPase aggregates at high concentrations, 100-fold diluted fractions were used for the Pma1p Westerns in *A* and *B*. (*D*) Exoglucanase activities in the two enzyme peaks shown in Fig. 2 are due to two different enzymes. Peak fractions were deglycosylated with Endo H (lanes *2* and *4*) or untreated (lanes *1* and *3*). Lanes *1* and *2*: Bgl2p peak fraction 9; lanes *3* and *4*: periplasmic enzyme peak fraction 18. Samples were analyzed by Western blot using polyclonal antiserum to Exg1p. The lack of abundant deglycosylated protein in lane *2* indicates that most of the exoglucanase activity in the Bgl2p peak fractions is likely due to another enzyme. Estimated molecular weights are indicated at the right.

tained in previous isolations of secretory vesicles (Walworth and Novick, 1987; Holcomb et al., 1987, 1988). This yield is sufficiently high so that a protein peak on SDS gels should be visible if invertase-containing organelles were the major accumulated membrane in the *sec6* mutant. The invertase activity as well as the exoglucanase activity in both peaks was sedimentable and releasable with 0.1% Triton X-100, and the majority of the activity was not detected if the assays were done in the presence of osmotic support and without the addition of Triton X-100 (not shown), indicating that the activities were membrane bound.

Holcomb et al. (1987, 1988) used a quite different method to isolate post-Golgi secretory vesicles accumulated by the *sec1* mutant. In their procedure, vesicles were accumulated after spheroplasting cells, and lysis was performed in a very different buffer by agitation with glass beads. They suggest that these conditions are important for preventing vesicle lysis. However, we obtained an almost identical yield of invertase activity in the P3 fraction as in our method when the cells were grown, spheroplasted, and lysed using their procedures (results not shown). Gradient fractionation of these P3 membranes yielded an SDS-PAGE gel protein pattern similar to that shown for *sec6* cells in Fig. 4. In addition, fractionation of *sec1* cells gave results similar to those seen for *sec6* (not shown).

Two Accumulated Membrane Populations Are Vesicles of Similar Size

Rather than a density gradient, Walworth and Novick (1987) used a Sephacryl S-1000 gel filtration column to separate secretory vesicles accumulated by *sec6* cells from other membranes present in the P3 fraction. We used this procedure to show that the various peak markers assayed in the Nycodenz gradient fractions elute from the S-1000 column in roughly the same peak, indicating organelles of similar size (Fig. 7). The markers for the less-dense vesicles elute slightly before the periplasmic enzyme peak, suggesting a slight size difference under our elution conditions. In our initial experiments in which a shorter column was used, the periplasmic enzyme and ATPase peaks coeluted, consistent with previous reports (Walworth and Novick, 1987). As was shown by gradient fractionation, these peaks are not detected in the column fractionation of wild-type cells (Fig. 7). Furthermore, SDS-PAGE of column fractions indicates that the peak of proteins cofractionating with the major ATPase activity detected in the gradient also coelutes with the enzyme peak from the column (Figs. 4 *A* and 8 *A*). Again, this protein peak is not seen in the SDS gel of wild-type fractions (Fig. 8 *B*). As for gradient fractionation of *sec6* cells, up to 90% of the acid-phosphatase activity applied to the column is recovered in the major periplasmic enzyme peak (fractions 25–38).

The ATPase profile of column fractions is different from that shown in previous reports (Walworth and Novick, 1987; Goud et al., 1988) which indicated a relatively much larger fraction of the activity in the void volume (fractions 21–24). The void-volume activity, also present in fractionation of material from wild-type cells, is likely due to plasma membrane, while the later peak, missing from wild-type cells, is enzyme carried by secretory vesicles. The difference between our and previous results is likely

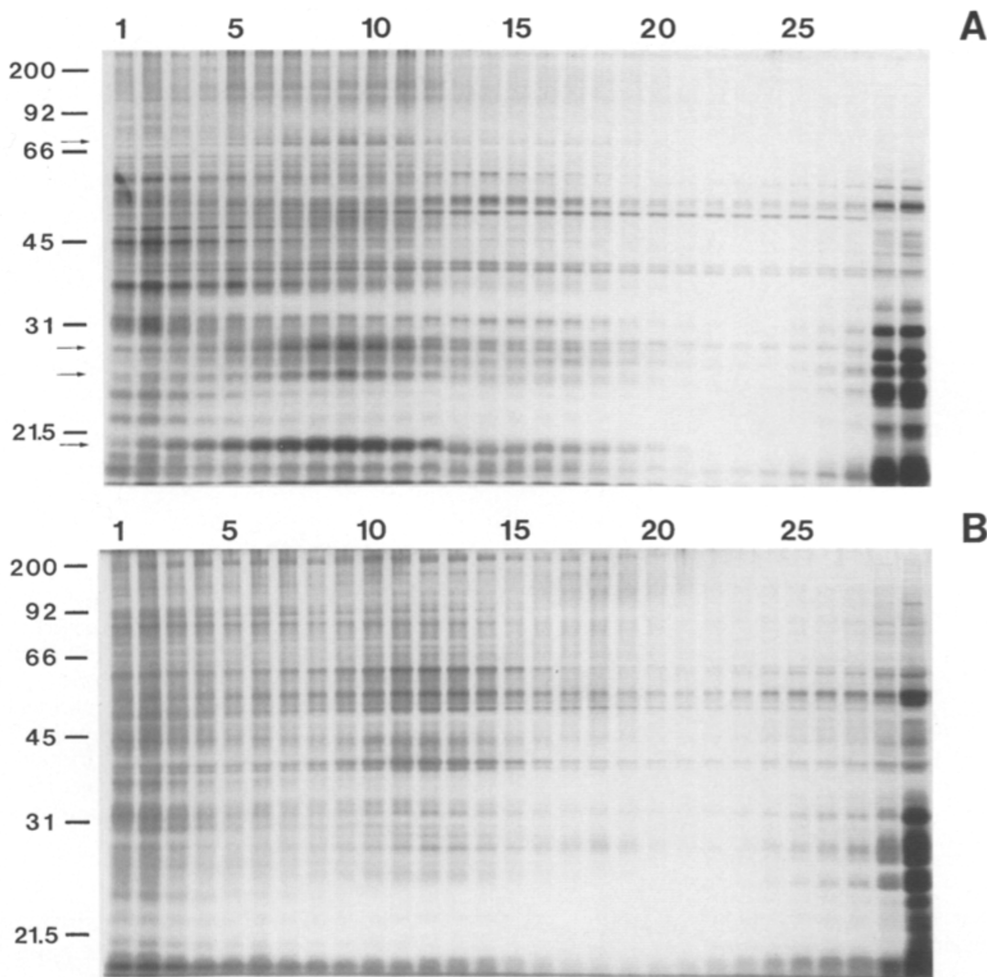

Figure 4. Polypeptide composition of Nycodenz gradient fractions from (*A*) *sec6* and (*B*) wild-type. Cells were fractionated as described in Figs. 1 and 2, and aliquots of each fraction (indicated at top) were resolved by SDS-PAGE and visualized by silver staining. Positions of molecular weight standards are shown on the left; polypeptides peaking with Bgl2p, with apparent molecular masses of 76, 29, 27, and 19 kD, are marked with arrows.

due to the higher level of glucose we added to the growth medium during vesicle accumulation (4% rather than 0.2%), which increases ATPase activity, and also possibly to the slightly harder centrifugation we used to obtain P2 (13,000 *g* for 20 min rather than 10,000 *g* for 10 min), which results in less plasma membrane contamination. Therefore, there is a relatively higher amount of ATPase activity in the secretory vesicle fractions.

Examination of the membrane peak fractions by electron microscopy also indicated vesicles of similar size. Thin sections of embedded membranes showed vesicles of ~100 nm diameter in both *sec6* gradient peaks (fractions 9 and 17, Fig. 9, *a* and *b*, respectively). Because the higher density vesicles are less abundant and peak close to a membrane peak also consistently present in wild-type gradients at a slightly lower density (fractions 11–16, with major bands having apparent molecular masses of 58 and 43 kD), the membranes in this contamination peak were also examined. As shown in Fig. 9 *c*, fraction 14 contained electron-dense tubular structures but very few lightly stained vesicles. While some of this material, possibly fragmented ER, is also present in the high-density vesicle peak (Fig. 9 *b*), the peak fraction (18) additionally contained lightly stained 100-nm vesicles. The vesicles in both peaks resemble those seen in thin sections of intact cells (Fig. 9 *g*). Additionally, negative staining showed the presence of smooth vesicles homogeneous in size and appearance in both the Bgl2p-containing and periplasmic enzyme peak fractions of the Nycodenz gradient, although some contamination from larger membranes was apparent in the periplasmic enzyme peak (Fig. 9, *d* and *e*). While equivalent fractions from wild-type cells also contained vesicles, they were more heterogeneous and much less abundant (Fig. 9 *f*).

Both Vesicle Populations Are Likely To Be post-Golgi Secretory Vesicles

The *sec6* mutant is known to accumulate late secretory vesicles but not ER or Golgi as was revealed by electron microscopy (Novick et al., 1980) and by the accumulation of Golgi-modified, but not ER forms of secreted glycopro-

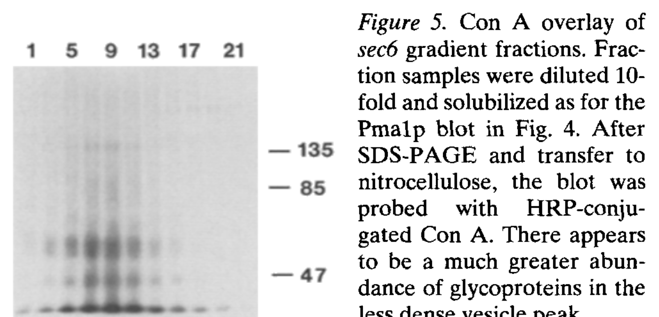

Figure 5. Con A overlay of *sec6* gradient fractions. Fraction samples were diluted 10-fold and solubilized as for the Pma1p blot in Fig. 4. After SDS-PAGE and transfer to nitrocellulose, the blot was probed with HRP-conjugated Con A. There appears to be a much greater abundance of glycoproteins in the less dense vesicle peak.

A

Fraction	Acid Phosphatase Activity	
	Units	% S1
Total	1.59	
P1	0.415	
S1	1.19	100
P2	0.249	20
S2	0.888	74
P3	0.369	31
S3	0.511	43
column pool	0.326	27
gradient pool	0.330	27

B

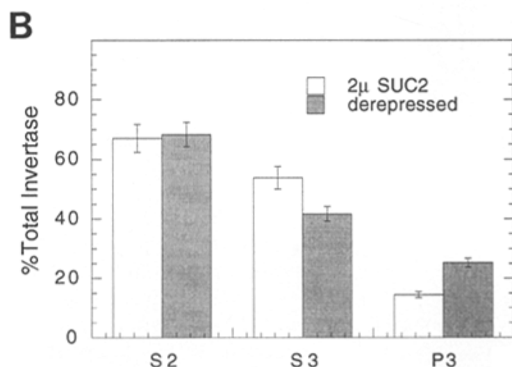

Figure 6. Distribution of periplasmic enzymes in fractionation of *sec6-4* cells. (*A*) Cells transformed with pDB31 were grown and fractionated as described in Fig. 1, and an aliquot from each fractionation step was assayed for acid phosphatase activity. (*B*) Cells were either transformed with pDB31 and grown as in Fig. 1 (*2μ SUC2*), or did not contain the plasmid and were shifted to low (0.2%) glucose, YP medium at the restrictive temperature rather than phosphate-depleted YP medium with 4% glucose (*derepressed*). An aliquot from each fractionation step was assayed for invertase activity. Bars are the means of four experiments.

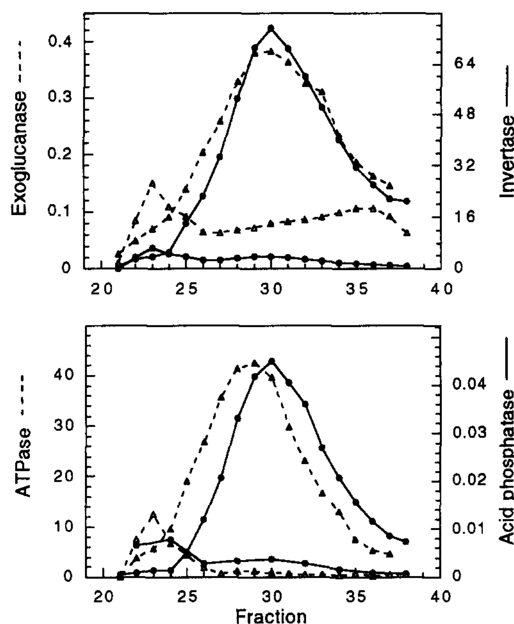

Figure 7. Enzyme activities in S-1000 column fractions from *sec6* and wild-type fractionation. *sec6-4* (*closed symbols*) or wild-type cells (*open symbols*) transformed with pDB31 were grown and fractionated as shown in Fig. 1. The P3 fraction was diluted to 2 ml and loaded onto a Sephacryl S-1000 column. 5-ml fractions were collected. Enzyme activities are indicated as units per fraction. The similar elution profile of the enzyme peaks from *sec6* fractionation indicates the presence of vesicles of similar size.

teins (Esmon et al., 1981). However, to eliminate the possibility that one of the enzyme peaks is due to the accumulation or fragmentation of another secretory organelle, we determined the location of ER, Golgi, and vacuole markers in gradient fractions. Enzyme assay for the Golgi marker guanosine diphosphatase (Abeijon et al., 1989; Yanagisawa et al., 1990) and Western blot for the ER marker Kar2p (Rose et al., 1989) showed that neither of these markers peak with either vesicle population (Figs. 2 and 3 *A*). A similar level of Kar2p was detected in gradients from wild-type cells (Fig. 3 *B*). Western blot for the vacuolar $[H^+]$ATPase, Vph1p (Manolson et al., 1992), indicated that vacuolar membranes peak at the top of the gradient rather than with either vesicle population (not shown). The guanosine diphosphatase activity in *sec6-4* gradient fractions was up to twofold higher than wild-type activity, suggesting some accumulation of Golgi in the *sec6-4* mutant. We were unable to detect Kex2p, a marker for late Golgi, in our gradients by Western blotting (not shown), which may be due to the low abundance of this protein in cells (Redding et al., 1991). However, Redding et al. have shown by gradient fractionation and immunofluorescence that this marker, detected by overexpression and a "sandwich-amplification" method, is normally distributed and does not accumulate in the late secretory mutant *sec1*. An experiment in which the surface proteins of spheroplasts were biotinylated before lysis and subsequently detected in gradient fractions with streptavidin–HRP by a blot overlay indicated that contaminating plasma membrane does not peak with either vesicle population (data not shown).

All the markers in the two peaks are either secreted enzymes or components of the cell surface believed to be transported by secretory vesicles, further indicating that both groups of accumulated membranes are likely to be secretory vesicles. Additionally, the same two peaks of vesicles were obtained when *sec4* or *sec1* cells were fractionated (not shown); these mutants are also blocked in the post-Golgi part of the secretory pathway and accumulate late secretory vesicles (Novick et al., 1980) and Golgi-modified glycoproteins (Esmon et al., 1981). The *sec6-4* mutant was chosen for most of our work because this mutant is tightly blocked at restrictive temperature and is most healthy at permissive temperature (Novick et al., 1980), minimizing the possibility of spontaneous suppression.

The *sec13-1* mutant, which is blocked early in the secretory pathway, accumulates ER but no secretory vesicles (Novick et al., 1980). As expected for vesicles derived from the secretory pathway, neither enzyme peak was apparent in fractionation of a *sec6 sec13* double mutant shifted to the restrictive temperature for 2 h (Fig. 10). Furthermore, the *sec18-1* mutant, also blocked early in the pathway (Novick et al., 1980), likewise did not accumulate either membrane populations found in the late *sec* mutants, as indicated by a silver-stained SDS-PAGE gel of gradient fractions (not shown) and exoglucanase assays (Fig. 10). It may be expected that glucanase activity above the levels detected in wild-type fractions should be present in this mutant, since ER and 50-nm vesicles are accumulated (Novick et al., 1980; Kaiser and Schekman, 1990).

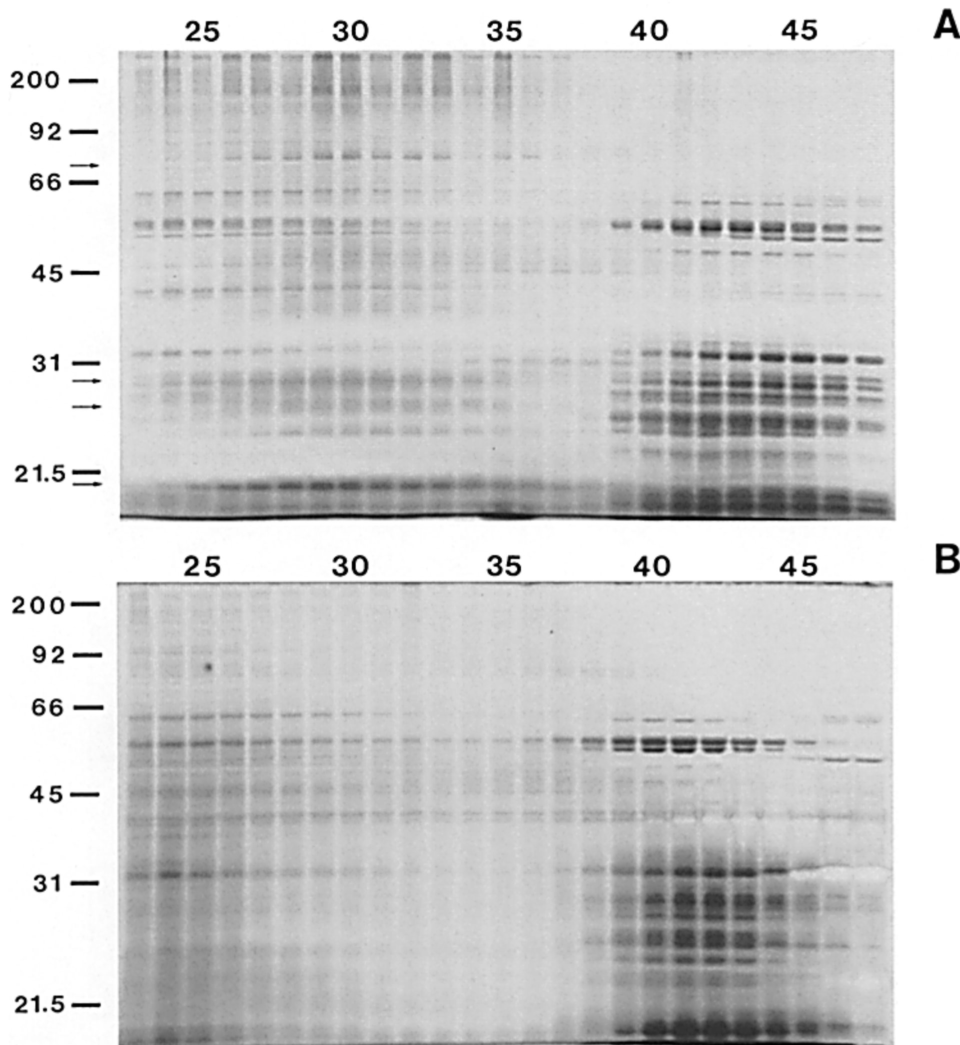

Figure 8. Polypeptide composition of Sephacryl S-1000 fractions from (*A*) *sec6* and (*B*) wild-type. Cells were fractionated as described in Figs. 1 and 7, and aliquots of fractions 23–47 were resolved by SDS-PAGE and visualized by silver staining. Lane *1* corresponds to fraction 23 in each panel. Positions of molecular weight standards are shown on the left; polypeptides coeluting with secretory vesicle markers, estimated as 76, 29, 27, and 19 kD, are marked with arrows.

However, most of the ER accumulated by *sec18* is likely to be removed by the low-speed spin, while the 50-nm vesicles are either inefficiently pelleted or not sufficiently abundant to be detected above background activity. Alternatively, Golgi modification may be necessary to detect the enzyme(s) present in the *sec6* vesicle peaks.

To further rule out the possibility that one vesicle population might be of endocytic origin, we examined a *sec6 end4* double mutant. Because End4p is thought to act specifically at the uptake step of endocytosis (Raths et al., 1993), the *end4* temperature-sensitive mutation should block the formation of endocytic vesicles at restrictive temperature. Enzyme assays and SDS-PAGE of gradient fractions (Fig. 11) indicate the presence of both vesicle populations in the double mutant, strongly arguing against either population being endocytic. Interestingly, the *end4* mutation alone results in the accumulation of the denser vesicle population, but no accumulation of the less dense vesicles.

Discussion

The secretory pathway in eukaryotic cells was initially defined in polarized pancreatic cells specialized for secretion (Jamieson and Palade, 1967*a*,*b*). By use of autoradiography, pulse–chase, and cell fractionation techniques, it was demonstrated that proteins transit to the cell surface through a series of membrane-bound organelles. Subsequent work has shown that steps in the processing of secretory proteins, such as the addition of oligosaccharide chains, are localized to specific compartments along the pathway (Dunphy and Rothman, 1985; Orci et al., 1985), and in examples of multiple species of plasma membrane–targeted vesicles, sorting into different vesicle populations usually occurs in a late Golgi compartment, the *trans*-Golgi network (Griffiths and Simons, 1986; Orci et al., 1987). The possibility of branching in the pathway to the cell surface in yeast has been hinted at previously (Novick and Schekman, 1983; Tschopp et al., 1984; Ballou et al., 1991; Liu and Bretscher, 1992; Bretscher et al., 1994; Govindan et al., 1995), and we now report the identification of two populations of post-Golgi secretory vesicles directed to the plasma membrane.

Using a combination of differential–velocity and Nycodenz density–gradient centrifugation, we have purified secretory vesicles accumulated in *sec* mutants defective in exocytosis. These vesicles were separated into two species based on their different densities. We reason that both vesicle species are destined for the plasma membrane because the contents of both populations are known to reach

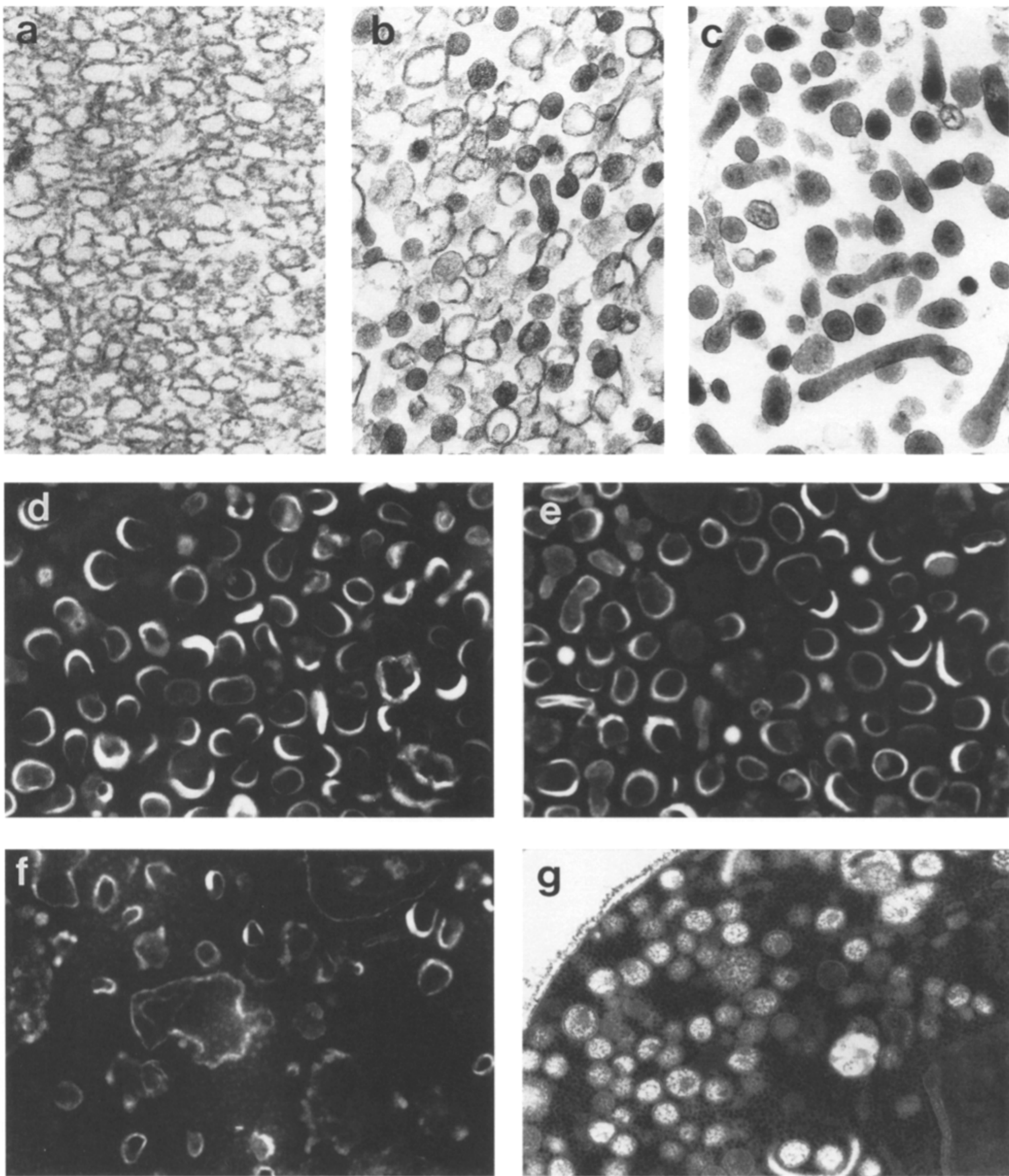

Figure 9. Electron micrographs of membranes in Nycodenz gradient fractions from *sec6-4* (*a–e*) and wild-type (*f*) cells, and in an intact *sec6-4* cell (*g*). Cells were fractionated as described in Figs. 1 and 2, and gradient fraction samples were prepared for thin section (*a–c*) or negative stain (*d–f*) electron microscopy. (*a* and *d*) Bgl2p peak (fraction 9); (*b* and *e*) invertase peak (fraction 18); (*c*) "contamination" peak (fraction 14); (*f*) wild-type fraction 9 (density of *sec6* Bgl2p peak). All micrographs are at the same magnification. Bar, 200 nm.

the cell surface via the secretory pathway, and because a *sec4* mutant, which has a defective Rab protein believed to act specifically in exocytosis, accumulates both vesicle populations. Mutants in which the secretory pathway is blocked at an early point, such as *sec18* and a *sec6 sec13* double mutant, do not accumulate either vesicle species. In addition, other organelle markers we assayed do not copurify with either vesicle population. Moreover, our re-

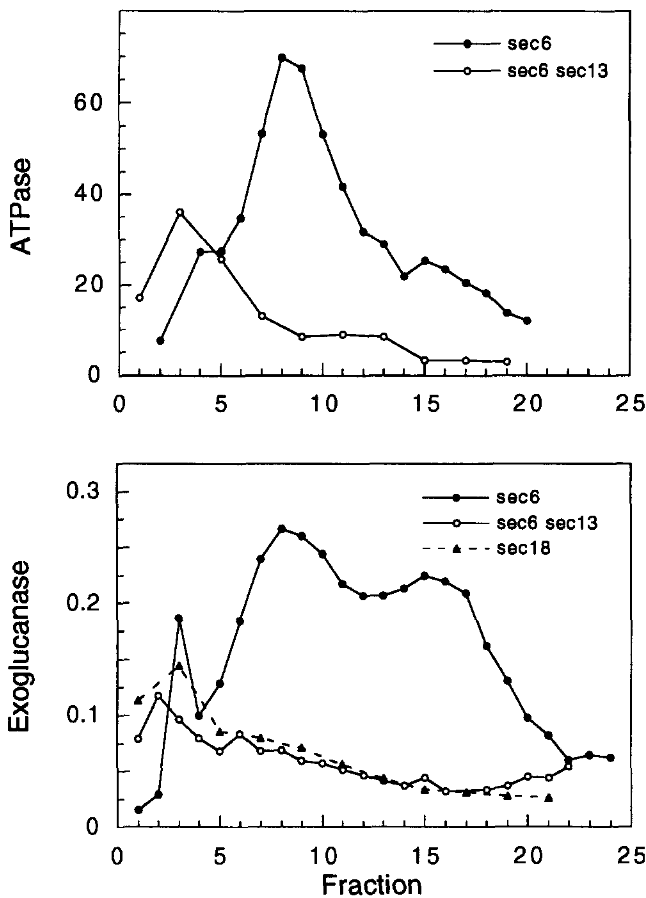

Figure 10. Enzyme activities in Nycodenz gradient fractions of the *sec18-1* and *sec6-4 sec13-1* double mutant, compared with activities present in *sec6-4* fractions. Cells were fractionated as in Figs. 1 and 2, and enzyme activities, shown as units per fraction, were determined. The absence of major exoglucanase and ATPase peaks in the *sec6 sec13* fractions and of exoglucanase in the *sec18* fractions indicates the absence of vesicles accumulated by *sec6* cells.

sult that both vesicle populations accumulate in a *sec6 end4* double mutant, expected to be blocked in the uptake step of endocytosis (Raths et al., 1993), further supports our conclusion that both vesicle species are intermediates in the exocytic pathway. The surprising finding that the *end4* mutation alone results in the accumulation of the more dense vesicles at the restrictive temperature, together with the knowledge that endocytosis is compromised in many late *sec* mutants (Reizman, 1985; Vida and Emr, 1995), suggests the existence of a compartment common to the endocytic and exocytic pathways. Further studies examining this possibility will be presented elsewhere. The accumulation of only one vesicle population in the *end4* mutant also indicates that neither of the two vesicle populations in *sec6* accumulate as an indirect result of blocking one pathway.

An explanation for two populations of post-Golgi vesicles is that one population, transporting materials for plasma membrane and cell-wall synthesis, such as the major plasma membrane ATPase, Pma1p, and the cell-wall component, Bgl2p, is specifically targeted to regions of active growth, while another population, carrying periplasmic enzymes and proteins secreted into the medium (invertase, acid phosphatase, Exg1p), does not need to be targeted so precisely. This model predicts that other secreted proteins shown to accumulate in *sec* mutants but not directly involved in surface expansion, such as the periplasmic enzyme α-galactosidase (Tschopp et al., 1984) and several plasma membrane permeases (sulfate, galactose, and arginine permease [Novick et al., 1980; Tschopp et al., 1984]) should be present in the invertase-containing vesicles but not in vesicles transporting Bgl2p.

Because it has been shown that acid-phosphatase secretion occurs mostly, although not exclusively, in the bud (Field and Schekman, 1980), periplasmic enzyme–containing vesicles may be transported to the bud but still be able to fuse at any location on the plasma membrane. This is suggested by the phenotypes of certain cytoskeletal mutants, such as *myo2-66*, a temperature-sensitive allele of a type V myosin (Johnston et al., 1991; Govindan et al., 1995) and *tpm1Δ*, a mutant lacking tropomyosin 1 (Liu and Bretscher, 1992). While these cells accumulate what appear to be post-Golgi secretory vesicles, they do not accumulate a significant amount of invertase. Perhaps a defective cytoskeleton results in inefficient transport of secretory vesicles (or other secretory organelles); vesicles that can fuse efficiently only at specific locations accumulate, unlike vesicles that can fuse anywhere on the plasma membrane. While the contents of vesicles in cytoskeletal mutants are yet to be identified, genetic evidence suggests that both Myo2p and Tpm1p are involved in the post-Golgi part of the secretory pathway (Liu and Bretscher, 1992; Govindan et al., 1995). Neither of the two vesicle populations we detected in late *sec* mutants appear to be the major population of vesicles accumulated by the *myo2* and *tpm1* mutants (our unpublished results).

Early studies of plasma membrane assembly in *sec* mutants have also suggested the possibility of divergence in the pathway to the cell surface (Tschopp et al., 1984). The delivery of several major plasma membrane proteins was shown to be much less defective in a conditional late *sec* mutant (*sec1*) than in mutants blocked at earlier steps (*sec7* and *sec18*), despite the fact that the delivery of other plasma membrane and periplasmic proteins is tightly blocked in both the early and late mutants (Novick et al., 1980; Novick and Schekman, 1983). While we found that *sec1* accumulates both of the vesicle species we have purified, this mutant may be more defective in the pathway transporting periplasmic enzymes. Alternatively, yet a third pathway may transport certain plasma membrane proteins.

If it is indeed the case that one vesicle population is more specifically targeted than the other, it should be very interesting to determine how the various components of the postulated membrane-recognition machinery are distributed among the two populations. Our results indicate that Snc1p, one of the two v-SNAREs believed to be on post-Golgi transport vesicles, is present on the Bgl2p-containing vesicles, although it is possible that this protein is also present on periplasmic enzyme–containing vesicles but is not detected due to the much lower abundance of this vesicle group. Protopopov et al. (1993) have suggested that Snc1p is on the invertase transport vesicles of *sec6* cells. However, this conclusion relied on the belief that in-

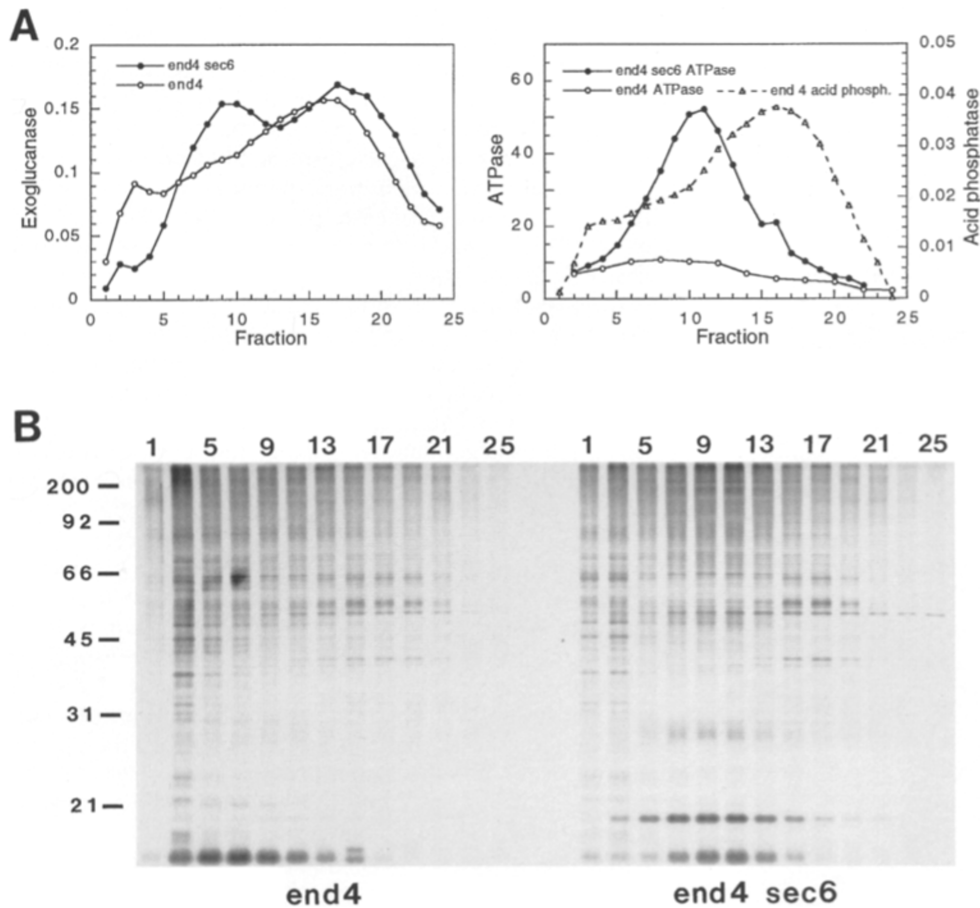

Figure 11. Enzyme activities and silver-stained SDS gel of Nycodenz gradient fractions from the *end4* and *end4 sec6* mutants. (*A*) Enzyme activities indicate that both the ATPase and exoglucanase peaks are present in the double mutant. The *end4* mutation alone results in the accumulation of markers for the denser vesicle population. (*B*) Silver-stained gel showing that the major membrane peak present in *sec6* cells is also found in the *sec6 end4* mutant. The *end4* mutant does not accumulate the vesicles represented by this peak. Only odd-numbered fractions were loaded, as indicated at the top.

vertase vesicles are the only population that accumulates in late *sec* mutants. While disruption of the genes encoding Snc1p and Snc2p resulted in some accumulation of invertase after a long shift to 37°C, this may be a secondary effect, as little invertase accumulation was seen at 30°C (Protopopov et al., 1993). It will be interesting to establish whether Snc1p and Snc2p are on different vesicles.

Determining which vesicle population(s) are associated with Sec4p may have important implications concerning the role of Rab GTP-binding proteins in regulating the specificity of membrane fusion. While much of the Sec4p in wild-type cells is on the plasma membrane, in late *sec* mutants such as *sec6*, a large portion of the Sec4p is sequestered on accumulated secretory vesicles (Goud et al., 1988). Our suspicion is that Sec4p is present on both vesicle species since both populations accumulate in the *sec4* mutant, although it is possible that one population accumulates as a secondary effect due to the sequestration of factors important for exocytosis. The presence of Sec4p on both vesicle species would not necessarily be inconsistent with a function for regulating the site on the plasma membrane where exocytosis takes place; in combination with other localizing factors, Sec4p could be involved in the fusion of both vesicle groups but not allow the fusion of targeted vesicles at inappropriate locations.

Multiple vesicle populations may also simply result from proteins being transported to the plasma membrane in vesicles budding from different compartments rather than sorting into different vesicles from the same compartment. This would imply that sorting occurs at multiple steps and a portion of secreted proteins bypass certain compartment(s), perhaps a post-Golgi intermediate compartment or a late Golgi compartment. While this suggestion may be considered a mild heresy in light of widely held beliefs about transport through the secretory pathway, it is not excluded by current knowledge of membrane trafficking in yeast, and it incorporates and provides functionality for multiple populations of post-Golgi vesicles. A bypassed compartment may, for example, contain glycosyl transferases. As carbohydrate is of higher density than protein (Esmon et al., 1981), vesicles with highly glycosylated cargo should be more dense than vesicles with less carbohydrate content. Further characterization of vesicle contents will determine whether the contents of one species tend to be more highly glycosylated. A bypassed compartment may also contain proteases or other modifying enzymes. While it may make sense that some proteins should avoid such a compartment, there is thus far no evidence to support this possibility.

There is, however, evidence suggesting that not all secreted proteins traverse the same glycosylating compartments. A screen for orthovanadate-resistant mutants has identified five complementation groups, all of which were defective in protein glycosylation and two of which were previously identified *mnn* (mannosylation) mutants (Ballou et al., 1991). Mutants in two of the complementation groups exhibited a defective glycosylation pattern of only a subset of secreted proteins, and Ballou et al. suggested

that different proteins might be processed through different compartments.

Other work has also suggested the possibility that secretory proteins do not all transit through the same compartments. Novick and Schekman (1983) have shown that newly synthesized proteins have a wide range of transit times through the secretory pathway. Some proteins take only 5 min or less to be secreted, while others may take longer than 30 min. The asynchrony was shown to develop before the final exocytic step and was suggested to be either because some proteins are transported through different compartments, or because the proteins move more slowly through the same pathway (Novick and Schekman, 1983). Our results are consistent with the first possibility, as the transit time of Pma1p is over 30 min (Chang and Slayman, 1991), while invertase is secreted in less than 5 min (Novick et al., 1981). It will be interesting to determine whether the other vesicle components follow a trend in transit times.

The first identification of yeast secretory mutants involved a screen for cells which accumulated and failed to secrete the periplasmic enzymes invertase and acid phosphatase (Novick and Schekman, 1979). It was noted that the two mutants isolated by this screen became more dense when shifted to restrictive temperature, and a subsequent screen which identified most of the known conditional–lethal mutations that block secretion involved an enrichment for mutants with increased density and screening for cells that accumulated periplasmic enzymes (Novick et al., 1980). Many mutants with increased density but no accumulation of periplasmic enzymes were not characterized further, and our results suggest that some of these mutants may have been specifically defective in the pathway transporting cell-surface components such as Bgl2p, with little effect on the secretion of periplasmic enzymes. Additionally, as mentioned by Novick et al. (1980), mutants defective in secretion of periplasmic enzymes but not in surface expansion (which may not increase in density) would not have been identified in this second screen. If periplasmic enzyme–containing vesicles are a minor population accumulated by mutants blocked in secretion, as our results indicate, then mutants specifically blocked in the transport of these enzymes may not have a significantly increased density.

Because the Bgl2p-containing vesicle population appears to be the major one accumulated by the three late *sec* mutants we examined, it is also possible that some of the *sec* mutants are directly blocked in the pathway transporting these vesicles but accumulate periplasmic enzyme–containing vesicles as a secondary effect. However, the phenotypes of the *myo2* and *tpm1* mutants suggest that cells are capable of accumulating vesicles without the accumulation of periplasmic enzymes. Therefore, the pioneering work of Novick et al. (1980) likely recovered just those mutants defective in exocytosis of all classes of post-Golgi transport vesicles. Additional attempts to isolate yeast secretory mutants may be fruitful in the investigation of how different cargoes are selected for each vesicle population and how the location and specificity of exocytosis is determined.

We are very grateful to Franz Klebl, Ruby Ye, Morris Manolson, Mark Rose, Rosario Cueva, Robert Fuller, and Ramon Serrano for antibodies, Peter Novick and Howard Riezman for yeast strains, and Randy Schekman for the *TPI-SUC2* plasmid. We also thank Marian Strang and Mark Berryman for advice on electron microscopy techniques, Bill Brown for use of equipment, and anonymous reviewers for helpful comments.

This work was supported by a grant from the National Institutes of Health (GM39006) and a National Institutes of Health Predoctoral Fellowship (GM07273).

Received for publication 30 March 1995 and in revised form 25 July 1995.

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