Secretory Vesicles Externalize the Major Plasma Membrane ATPase in Yeast

Cherie L. Holcomb, William J. Hansen,* Tina Etcheverry,‡ and Randy Schekman

Department of Biochemistry, University of California, Berkeley, California 94720;
* Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143;
and ‡ Genentech, South San Francisco, California 94080

Abstract. Yeast cell surface growth is accomplished by constitutive secretion and plasma membrane assembly, culminating in the fusion of vesicles with the bud membrane. Coordination of secretion and membrane assembly has been investigated by examining the biogenesis of plasma membrane ATPase (PM ATPase) in secretion-defective (*sec*) strains of *Saccharomyces cerevisiae*. PM ATPase is synthesized as a ~106-kD polypeptide that is not detectably modified by asparagine-linked glycosylation or proteolysis during transit to the plasma membrane. Export of the PM ATPase requires the secretory pathway. In *sec1*, a mutant defective in the last step of secretion, large amounts of Golgi-derived vesicles are accumulated. Biochemical characterization of this organelle has demonstrated that PM ATPase and the secretory enzyme, acid phosphatase, are transported in a single vesicle species.

THE secretory pathway has been well characterized in mammalian cells (21, 30). Cell surface proteins are synthesized at the rough endoplasmic reticulum (ER)[1], transit the Golgi apparatus, and enter secretory vesicles that fuse with the plasma membrane, externalizing their contents.

Previous investigations from our laboratory have revealed that a secretory pathway, homologous to that in mammalian cells, exists in the yeast *Saccharomyces cerevisiae* (26, 28, 29). Such evidence has been obtained by the identification and characterization of secretion (*sec*) mutants. These strains contain conditionally lethal mutations that block secretion at the restrictive temperature causing accumulation of proteins at specific stages of secretion: *sec61*, before translocation across the ER membrane (5); *sec53*, before glycosylation in the ER lumen (9, 10); *sec18*, in the ER (28); *sec7*, in the Golgi apparatus (29); *sec1*, at the stage of secretory vesicles (26).

Secretion and plasma membrane assembly in yeast are accomplished by constitutive transport. The export of both secretory and plasma membrane proteins is blocked in all *sec* mutants (27, 29, 43). This result is consistent with two possibilities: (*a*) plasma membrane proteins and secretory proteins may be transported to the cell surface in the same vesicles; (*b*) these two types of protein may be sorted away from each other and transported in separate vesicles that both require the *SEC* gene products for fusion with the plasma membrane.

An observation made by Atkinson and Ramirez (1) suggests that secretion and plasma membrane assembly may be uncoupled. When yeast inositol-requiring mutants are starved for inositol and grown in media containing a nonfermentable carbon source, secretion can be detected without accompanying net cell surface growth. One interpretation of this result is that a single vesicle is responsible for all surface assembly, and inositol starvation enhances the rate of plasma membrane turnover. Alternatively, plasma membrane proteins may populate a subset of Golgi-derived vesicles distinct from those containing secretory proteins, and phosphatidylinositol (or some derivative) may be required for export of the former but not the latter vesicle species.

Regulated secretion in mammalian endocrine and exocrine cells provides a clear case for multiple species of vesicles involved in export (18). AtT20 pituitary cells, for example, secrete ACTH and other artificially introduced regulated proteins in a secretagogue-stimulated fashion, whereas two viral glycoproteins and other constitutively secreted proteins are transported continuously and rapidly (13, 23, 24). Analysis of purified ACTH-containing granules has shown conclusively that the two classes of protein are transported by distinct mature vesicles (13). However, it has not been possible to determine if there is a single vesicle population or multiple vesicle types involved in constitutive secretion.

A distinct subpopulation of clathrin-coated vesicles has been identified that carries synaptic vesicle proteins (31). Immune adsorption was used to demonstrate that within a preparation of physically homogeneous particles, multiple vesicle membrane varieties may be represented. In this case the coated vesicles may contain intermediates in the assembly of several organelles.

1. *Abbreviations used in this paper*: CoxIV, cytochrome *c* oxidase subunit IV; DPAPase, dipeptidylaminopeptidase; ER, endoplasmic reticulum; HS, high speed; LE, liquid electrophoresis; LS, low speed, PG, Percoll gradient; PM, plasma membrane; *sec*, secretion mutant; TBS, Tris-buffered saline.

Table I. Yeast Strains Used

Strain	Genotype	Source or reference
X2180-1A	*MATa gal2−*	YGSC*
HMSF176	*MATα sec18-1*	7
SF263-1A	*MATa sec1-1*	7
SF294-1C	*MATα sec7-1*	7
SF300-2D	*MATa sec53-6*	9
ISY1-8B	*MATα sec1-1 pho80*	I. Schauer

* Yeast Genetic Stock Center, University of California, Berkeley, CA.

Membrane protein assembly in polarized epithelial cells represents an independent case where distinct vesicles are involved in transport to separate regions of the plasma membrane (38). Although these vesicles have not been isolated, their existence has been deduced through analysis of the biogenesis of influenza hemagglutinin, which is transported to the apical surface, and vesicular stomatitis virus (VSV) G protein, which is assembled on the basolateral surface (33). It is clear that both proteins move through the same Golgi membrane stacks, diverge from each other possibly in the trans-Golgi network, and are assembled separately in the respective surface domains in a constitutive process (12, 32).

The yeast secretory mutant *sec1* accumulates large quantities of the vesicles that mediate export of secretory and plasma membrane proteins (11, 26). To determine if multiple vesicle types are present, a procedure has been devised for the partial purification of these Golgi-derived vesicles. We have followed the biogenesis and export of a major plasma membrane protein, the vanadate-sensitive Mg^{2+}-ATPase (36, 37), and analyzed the vesicles for the coincidence of this plasma membrane (PM) ATPase and secretory proteins.

Materials and Methods

Strains, Plasmids, Growth Conditions, and Materials

The yeast strains used in this study are listed in Table I.

Rich and minimal growth media were as before (5). Optical density of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm in a model DUR spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) fitted with a photomultiplier (Gilford Instrument Laboratories, Inc., Oberlin, OH), 1 OD_{600} unit equals 1 ml of cells with an OD_{600} of 1. All experiments were initiated with cells in the exponential phase of growth.

Reagents were obtained as indicated. Percoll was from Pharmacia, Uppsala, Sweden; carrier-free $H_2{}^{35}SO_4$ (40 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, IL; IgG Sorb was from the Enzyme Center, Inc., Boston, MA; nitrocellulose was from Schleicher & Schuell, Inc., Keene, NH; antisera directed against acid phosphatase, cytochrome *c* oxidase subunit IV, and hexokinase were generously provided by G. Schatz, Biozentrum, University of Basel; plasma membrane PM ATPase antiserum and antiserum directed against the 30-kD fragment of the PM ATPase were the gift of N. Nelson, Dept. of Biochemistry, Roche Institute of Molecular Biology, Nutley, NJ; endoglycosidase H (endo H) was generously provided by P. Robbins, Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA. Lyticase (fraction II) was purified as described previously (35). Iodination of protein A was performed by the chloramine T/NaI method (16). All other reagents were of the highest available purity and were obtained from Sigma Chemical Co., St. Louis, MO.

Preparation and Radiolabeling of Spheroplasts

Spheroplasts were prepared as described elsewhere (15) except that cells were grown in minimal medium to an OD_{600} of 0.5 and spheroplasting medium contained 0.7 M sorbitol + minimal medium adjusted to pH 7.4 with potassium phosphate.

Metabolic labeling was performed with spheroplasts prepared from cells grown in minimal medium containing chloride salts in place of sulfate salts, supplemented with 100 μM $(NH_4)_2SO_4$ and 2% glucose. Before labeling, spheroplasts were centrifuged (1,000 *g*, 5 min), washed twice at 100 OD_{600} U/ml in labeling medium (sulfate-free minimal + 20 μM $(NH_4)_2SO_4$ + 0.7 M sorbitol), and resuspended in fresh labeling medium at 50 OD_{600} U/ml. After a 30-min shift to 37°C to insure complete imposition of the *sec* mutant block, $H_2{}^{35}SO_4$ was added (50 μCi/OD_{600} unit) and labeling was carried out for 60 min. At the end of the labeing period, NaN_3 and $(NH_4)_2SO_4$ were added to a final concentration of 10 mM and 1 mM, respectively, and samples were chilled quickly on ice.

Secretory vesicles were isolated from radiolabeled spheroplasts by the same procedure developed for their isolation from cells grown in rich medium (15).

Enzyme Assays

Acid phosphatase was assayed by the method of Vin Rijn et al. (44). NADPH cytochrome *c* reductase was assayed as described by Kubota et al. (20). α,1-3Mannosyl transferase was assayed by the method of Nakajima and Ballou (25). Assays for heat stable (DPAPase A) and heat labile (DPAPase B) dipeptidylaminopeptidase were performed as described elsewhere (3, 17) except that the reaction was stopped by addition of TCA to a final concentration of 4%. The resulting precipitate was removed by centrifugation at 12,000 *g* for 15 min. Protein was measured by the Bradford procedure as modified by Vincent and Nadiau (45). This procedure decreases interference by Percoll and increases detection of membrane proteins. Bovine serum albumin was used as a standard. Cytochrome *c* oxidase subunit IV (CoxIV) and hexokinase were quantified by immunoblotting.

Immunoprecipitations and Immunoblotting

Immunoprecipitations were performed as described by Schauer et al. (34) with the following modifications. To minimize heat-induced aggregation of PM ATPase, samples were dissociated both before and after immunoprecipitation at 50°C for 15 min. The SDS concentration of the samples was adjusted to 0.4% before the addition of antibody to reduce nonspecific background. Unless otherwise stated, PM ATPase and acid phosphatase were immunoprecipitated from an equivalent of 0.13 and 0.25 OD_{600} unit of cell equivalents, respectively. All immunoprecipitations were done in antibody excess (4 μl of ATPase serum/OD_{600} unit; 30 μl of acid phosphatase serum/OD_{600} unit). Samples resolved by SDS-PAGE were fixed, treated with 1 M sodium salycilate for 20 min, dried, and visualized by autoradiography on Kodak X-Omat AR film at −70°C. Autoradiograms were quantified by scanning with a SD3000 spectrodensitometer coupled to a SD300 density computer (Kratos Analytical Instruments, Ramsey, NJ) and 338 A integrator (Hewlett-Packard Co., Palo Alto, CA).

Immunoblotting was performed as previously described (2) except that all washes and incubations were done in TBS + 0.1% Nonidet P-40 and 2% nonfat dry milk (purchased in a local grocery store).

Competition by nonradioactive PM ATPase for immune precipitation of radiolabeled protein was performed with PM ATPase provided by G. Hammes, Section of Biochemistry, Cornell University, Ithaca, NY prepared as described (19). Further purification of this material was performed by SDS-PAGE on a 6% gel. After Coomassie staining, the PM ATPase band was excised and soaked in 100 vol of water changed three times over 4 h, then pulverized. Gel pieces contained ~10 μg PM ATPase/0.15 ml of gel. Control incubations included gel pieces containing no PM ATPase. Competition was performed with gel pieces because once embedded in polyacrylamide, the ATPase could not be eluted. Nevertheless, this method proved convenient and reproducible.

For immune precipitation of secretory vesicles, PM ATPase or control (nonimmune) antiserum was first complexed with IgG Sorb (fixed *Staphylococcus aureus* cells) by incubating 10 μl of antiserum with 200 μl of a 10% suspension of IgG Sorb in PBS, 0.5% Triton × 100, 1 mg/ml BSA for 16 h at 4°C. IgG Sorb complexes were collected by centrifugation (12,000 *g*, 1 min), washed twice in 1 ml of lysis buffer (5 mM Mes, pH 6.5, 0.7 M sorbitol, 5 mM $MgSO_4$, 5 mM NaN_3), and resuspended in 100 μl of the same buffer. This antibody-IgG Sorb conjugate (40 μl) was used to immune precipitate secretory vesicles from 0.25 OD_{600} unit of cell equivalents. The total volume was adjusted to 0.5 ml with lysis buffer, saponin (0.01%) or 0.25 mg/ml BSA was added to reduce nonspecific association of proteins with the IgG Sorb. Secretory vesicles retained sedimentable acid phosphatase in either buffer. Samples were incubated for 12 h at 4°C. The IgG Sorb conjugate, including bound vesicles, was collected by centrifugation at 12,000 *g*, 15 s in a microfuge. Supernatant fractions containing uncomplexed

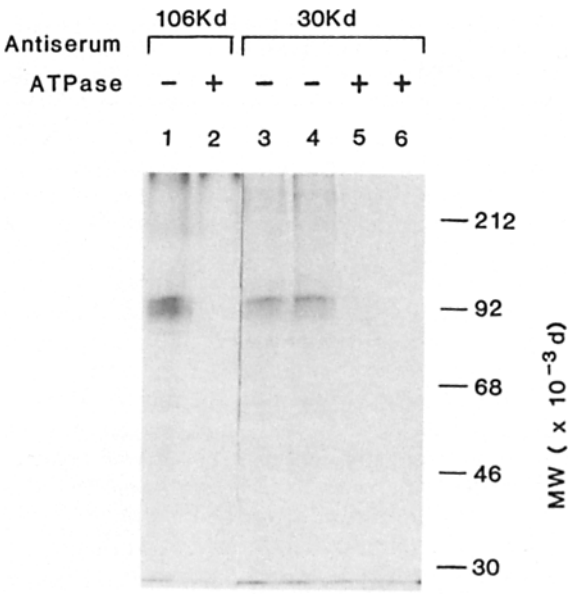

Figure 1. Immune precipitation of radiolabeled ATPase. X2180 cells were converted to spheroplasts, radiolabeled for 90 min with $H_2{}^{35}SO_4$, washed as before (15), and lysed in 1% SDS. Aliquots of solubilized lysate corresponding to 0.13 OD_{600} unit of cells were treated with antiserum (raised to the 106-KD species or 30-kD fragment of ATPase) in the presence or absence of nonradioactive ATPase as described in Methods. (Lane *1*) 150 μl of polyacrylamide gel added to immune precipitation; (lane *2*) same as lane *1* + 10 μg of nonradioactive ATPase; (lane *3*) no additions; (lane *4*) same as lane *1*; (lane *5*) same as lane *1* + 1 μg nonradioactive ATPase; (lane *6*) same as lane *2*. Precipitated material was solubilized and resolved by SDS-PAGE on a 6% gel.

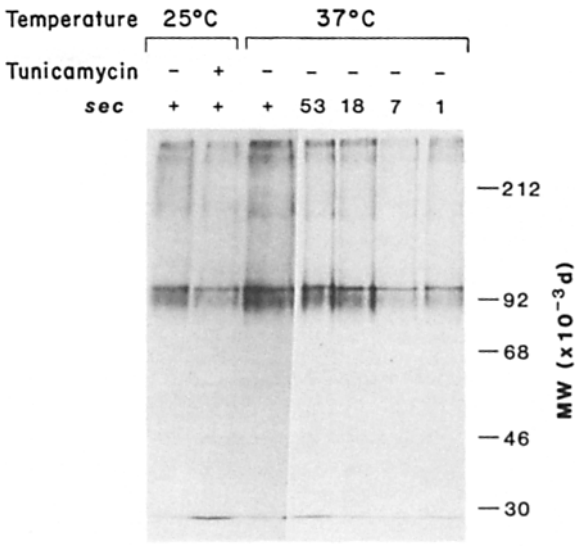

Figure 2. 106 kD ATPase is made in wild-type and *sec* mutant cells. Spheroplasts (strains X2180, SF300-2D, HMSF176, SF294-1C, SF263-1A) were radiolabeled at various temperatures as described in Materials and Methods except that labeling was performed at 6 OD_{600} unit of cell equivalents/ml with 150 μCi $^{35}SO_4{}^{2-}/OD_{600}$ unit. Where indicated, tunicamycin was added to 10 μg/ml at the beginning of the 30-min preincubation. Labeled spheroplasts were washed and lysed in 1% SDS. ATPase was immune precipitated from 0.13 OD_{600} unit of cell equivalents (*SEC*$^+$ samples) or 0.25 OD_{600} unit (*sec* mutant samples). Approximately equal levels of radiolabeled ATPase were detected in all samples. Lanes *1–3* were from the same gel as lanes *4–7* but were exposed to x-ray film longer to compensate for the smaller sample size.

proteins were transferred to new tubes for analysis. To dissociate bound proteins from IgG Sorb, the fractions were incubated in 0.25 ml of 1% SDS for 15 min at 50°C. IgG Sorb was removed by sedimentation and discarded. The resulting supernatant fractions, containing proteins that had previously been bound to the IgG Sorb, were saved for analysis.

Results

ATPase Biogenesis Monitored in Wild-Type and sec Mutant Cells

To assess the role of secretory organelles in the transport of a plasma membrane protein to the cell surface, we examined the biogenesis of the PM vanadate-sensitive Mg^{2+}-ATPase. Polyclonal rabbit antiserum raised in response to the purified protein was used to immune precipitate PM ATPase from radiolabeled spheroplasts. SDS-PAGE of immune precipitates (Fig. 1) revealed a ~106-kD polypeptide whose recovery was diminished specifically by competition with purified nonradioactive PM ATPase (lanes *1* and *2*). The antiserum reacted with major proteolytic fragments of the purified protein (Nelson, N., personal communication). Antiserum raised against a purified 30-kD fragment of the PM ATPase also recognized the 106-kD polypeptide (lanes *3* and *4*). This reaction was also diminished by competition with the intact protein (lanes *5* and *6*).

Preliminary experiments in which plasma membrane fractions were isolated from *sec* mutant cells radiolabeled at 37°C (restrictive temperature) showed that export of PM ATPase was reduced fourfold in *sec* mutant cells relative to wild type cells (14). Hence, as with other plasma membrane proteins (26, 27, 43), PM ATPase externalization appeared to require the *SEC* gene products.

Many secretory proteins experience proteolytic processing or addition of asparagine-linked oligosaccharide during intracellular transport. When secretion is blocked in *sec* mutant cells these processing events are interrupted, causing precursor proteins of distinct electrophoretic mobility to accumulate (7, 39). To determine if PM ATPase is subject to processing, we examined the forms of this protein accumulated in *sec* mutant cells at 37°C where assembly of secretory proteins in the ER (*sec53*), transport from the ER (*sec18*), transport within the Golgi body (*sec7*), or transport of secretory vesicles (*sec1*) was blocked. Fig. 2 shows that the same ~106-kD species was radiolabeled in wild type cells and in mutant cells at 37°C. Furthermore, no mobility shift was detected in PM ATPase synthesized in the presence of tunicamycin, an inhibitor of asparagine-linked glycosylation (Fig. 2, lane *2*). A predicted transmembrane orientation of the PM ATPase based on sequence analysis of the gene suggested a single asparagine glycosylation site on the external surface of the membrane (37). Evidently, this site was not used because our results indicate that the PM ATPase acquired no significant mobility-perturbing modifications during biogenesis. Given the size of the ATPase, however, minor changes in SDS-gel mobility may not have been detected.

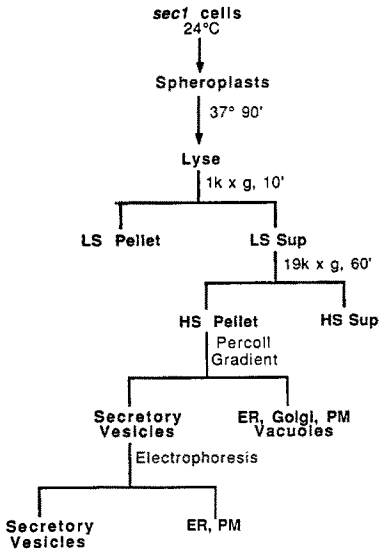

Figure 3. Outline of secretory vesicle isolation. *LS*, low speed; *HS*, high speed; *ER*, endoplasmic reticulum; *PM*, plasma membrane.

Table II. Markers for Cellular Compartments

Marker	Compartment
Acid phosphatase	Secretory vesicles
Hexokinase	Cytoplasm
NADPH cytochrome *c* reductase	ER
Mannosyl α,1-3 transferase	Golgi apparatus
DPAPase B	Vacuole
CoxIV	Mitochondria (inner membrane)

Newly Synthesized ATPase in Secretory Vesicles

A test of the hypothesis that plasma membrane and secretory proteins are transported to the cell surface in the same Golgi-derived vesicles required the isolation of secretory vesicles in high yield and purity. Unfortunately, wild type cells have a low steady-state pool of these secretory organelles (26). For this reason we used *sec1* cells which accumulate a large pool of vesicles at 37°C. These vesicles resemble those of wild type cells in electron micrographs of yeast thin sections and are competent for fusion with the plasma membrane upon return of the *sec1* cells to 24°C (permissive temperature) (26).

During the purification secretory vesicles were followed by their content of the secretory protein acid phosphatase (26). Continuous production of this enzyme was insured by use of the *pho80* mutation (42) which allows constitutive expression of the *PHO5* (acid phosphatase) gene. *sec1 pho80* cells were converted to spheroplasts so that acid phosphatase that had been secreted was released. The spheroplasts were then incubated at 37°C to accumulate vesicles containing acid phosphatase. A procedure for the isolation of the vesicles is shown in Fig. 3, and described in detail elsewhere (15). The procedure was optimized for recovery of acid phosphatase in a sedimentable form (i.e., enclosed within vesicles) and for maximum resolution of this enzyme from marker proteins that represented other cellular compartments (Table II). Differences in rate of sedimentation, density on a Percoll gradient, and electrophoretic mobility were exploited to achieve purification. Secretory vesicle preparations proved to be enriched for acid phosphatase by at least sixfold over every other major cellular membrane marker (reference 15, and Table III).

Secretory vesicles sedimented at a high density (1.09 g/cc) on Percoll gradients. Fig. 4 shows a typical profile in which acid phosphatase and NADPH cytochrome *c* reductase (ER) activities, and cytochrome *c* oxidase subunit IV (CoxIV, mitochondrial inner membrane) immunoreactivity were assayed. A low density peak which contained most of the ER marker and about half of the mitochondrial marker also contained the bulk of the DPAPase B (vacuolar membrane) (3), α,1-3 mannosyl transferase (Golgi membrane) (8), and ^{125}I-labeled plasma membrane (15) (not shown). Assay of the mitochondrial matrix enzyme fumarase showed significant release into the soluble fraction after lysis of spheroplasts (not shown). Hence, the bimodal distribution of CoxIV protein may have reflected intact mitochondria in the denser fractions and membrane fragments in the lighter fractions.

Secretory vesicles migrated more rapidly than other membranes during electrophoretic separation. Electrophoresis was performed through a column of liquid that contained a gradient of Ficoll to prevent convection currents (15). A typical pattern, shown in Fig. 5, displays the electrophoretic behavior of ER, mitochondrial membranes, and secretory vesicles obtained from the pooled, acid phosphatase-containing

Table III. Purification of Secretory Vesicles

	Relative specific activity (% recovery)*					
Marker	Acid phosphatase	Hexokinase	NADPH cytochrome *c* reductase	α, 1-3 Mannosyl transferase‡	DPAPaseB	CoxIV
Homogenate	1.0 (100)	1.0 (100)	1.0 (100)	–	1.0 (100)	1.0 (100)
LS sup	1.0 (39)	1.4 (95)	0.5 (20)	–	0.8 (23)	0.4 (11)
HS pellet	4.3 (22)	0.5 (7)	2.3 (15)	1.0 (100)	3.0 (12)	1.9 (10)
PG vesicles§	10.3 (12)	0.07 (0.2)	0.7 (0.8)	0.5 (17)	2.8 (3)	2.2 (3)
LE vesicles‖	24.0 (9)	0.08 (0.06)	0.7 (0.1)	0.9 (13)	4.0 (0.8)	3.4 (1.4)

* Based on preparation of secretory vesicles from cells grown on minimal media.
‡ Yeast cytosol contains an activity which interferes with detection of α, 1-3 mannosyl transferase activity. Therefore, values for this enzyme are expressed relative to the activity in HS pellet.
§ Vesicles purified by Percoll gradient.
‖ Vesicles purified by liquid electrophoresis.

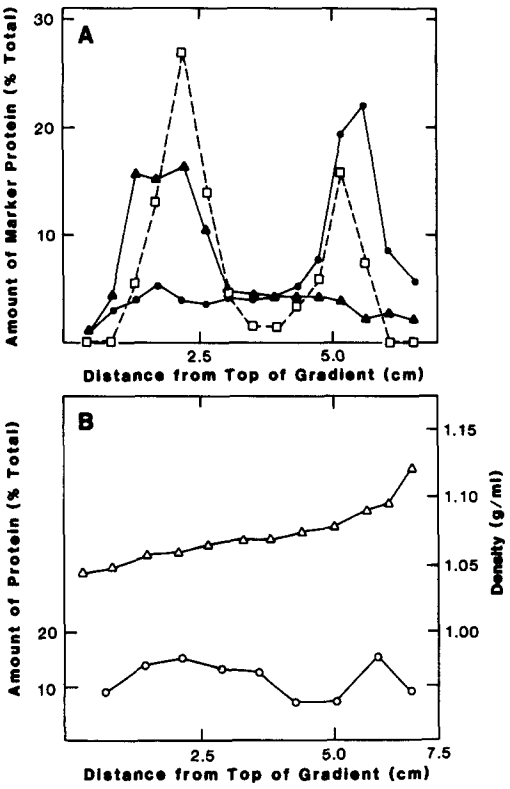

Figure 4. Percoll gradient fractionation. Membranes in a HS pellet fraction were applied to a Percoll gradient as described elsewhere (15). (*A*) Fractions were assayed for acid phosphatase (-●-) and NADPH cytochrome *c* reductase (-▲-) activities and were immunoblotted for CoxIV (- - - □ - - -). (*B*) Density, measured by weighing fixed volumes (-△-) and total protein profiles (-○-) of the same gradient. Results are expressed as percent of the total protein or activity applied to the gradient.

fractions of a Percoll density gradient. The peak containing CoxIV also contained the majority of the ^{125}I-labeled plasma membranes and DPAPase B activity (data not shown).

A summary of the recovery and enrichment of acid phosphatase and of other marker proteins is shown in Table III.

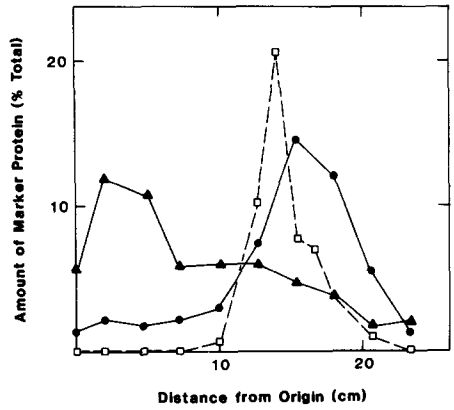

Figure 5. Liquid electrophoretic fractionation. A pool of Percoll gradient purified secretory vesicles were further resolved by electrophoresis on a Ficoll gradient (15). Assays and data treatment are as in Fig. 4.

These data were obtained from fractionation of cells grown in minimal growth medium. Results obtained with rich growth medium (15) differ primarily in that a larger purification factor of acid phosphastase was achieved in minimal medium (24-fold vs. 13-fold). This would be expected if cells grown in rich medium accumulated more secretory vesicles because the protein contained within the secretory vesicles would then represent a greater proportion of the total cellular protein. Thus, the purity of these preparations may be similar.

To determine if PM ATPase was present in the secretory vesicles, we examined fractionation of newly synthesized PM ATPase and acid phosphatase by immune precipitation of samples obtained from pulse radiolabeled spheroplasts. Recovery and purification of these two proteins are shown in Table IV. Profiles of the Percoll density gradient and liquid electrophoresis steps are shown in Fig. 6. Whereas other membrane markers were resolved as before, the radioactive PM ATPase and acid phosphatase fractionated concidentally. These data suggested that the two proteins shared a common carrier vesicle. However, it was possible that two different vesicle populations were present in these preparations and cofractionated on the basis of remarkably similar size, density, and charge.

Secretory Vesicles Precipitated by ATPase Antibody

Colocalization of acid phosphatase and PM ATPase was further investigated by direct isolation of vesicles containing PM ATPase. Such isolation was achieved by immune precipitation of purified vesicles with antibodies that recognize cytoplasmically oriented domains of the PM ATPase (36). This approach was used by Pfeffer and Kelly to demonstrate a subpopulation of coated vesicles that contained synaptic vesicle antigens (31). Under conditions that maintained vesicle integrity, radiolabeled vesicles (purified through the Percoll gradient step) were treated with PM ATPase or nonimmune antibodies conjugated to IgG Sorb. The PM ATPase and acid phosphatase contained in vesicles that were complexed to this IgG Sorb were quantified. Fig. 7 shows that both proteins were recovered quantitatively in the bound fraction when vesicles were treated with ATPase antibody, whereas both proteins remained in the free fraction when control antibodies were used. Once again, these data suggest that the two proteins are present in the same vesicle.

One potential artifact was that coprecipitation of acid phosphatase and PM ATPase resulted from aggregation of different membranes during the course of the experiment. In this case, ATPase exposed on the surface of a membrane would have caused the aggregate to bind to IgG Sorb causing proteins to coprecipitate nonspecifically. This possibility was

Table IV. Copurification of [^{35}S]ATPase and Secretory Vesicles

	Specific activity (and % yield) in fractions	
	Acid phosphatase-SV	[^{35}S]ATPase
Homogenate	1	1
LS sup	1 (100)	0.8 (100)
HS pellet	4 (70)	4 (67)
PG vesicles	31 (37)	25 (31)

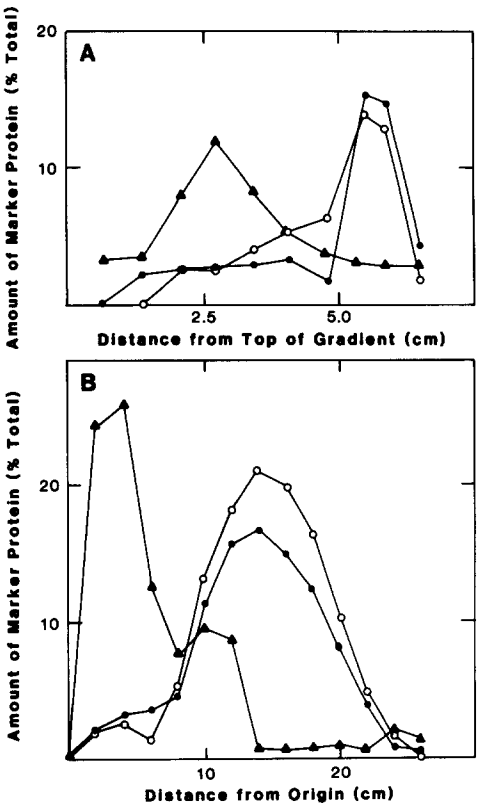

Figure 6. Newly synthesized ATPase cofractionates with secretory vesicles. Secretory vesicles were isolated from $^{35}SO_4^{2-}$-labeled spheroplasts (ISY1-8B). Samples were assayed for NADPH cytochrome *c* reductase activity. Newly synthesized ATPase and acid phosphatase were immune precipitated, resolved by SDS-PAGE, and quantified by densitometric scanning of autoradiograms. Profiles of a Percoll gradient (*A*) and liquid electrophoretic separation (*B*) are plotted using symbols as in Figs. 4 and 5; ATPase (-o-). Data are expressed as described in Fig. 4.

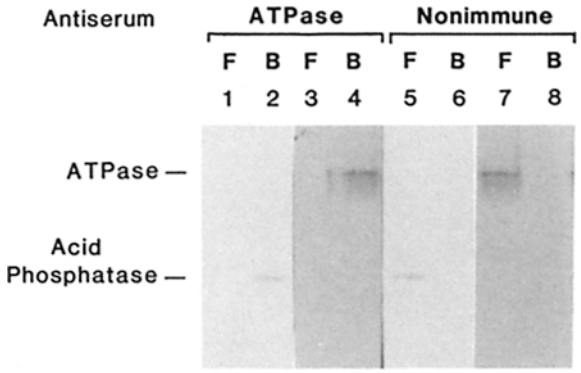

Figure 7. Acid phosphatase and ATPase are coprecipitated from vesicle preparations. Under conditions that maintained the integrity of vesicles, Percoll gradient-purified vesicles from $^{35}SO_4$-labeled spheroplasts were mixed with IgG Sorb that had been treated with nonimmune serum (lanes *5–8*) or with ATPase antiserum (lanes *1–4*) as described in Materials and Methods. Briefly, IgG Sorb conjugates, including bound proteins, were collected by very low speed centrifugation at a speed where vesicles did not sediment. Supernatant fractions containing uncomplexed (free, *F*) proteins were transferred to new tubes for analysis. Proteins were dissociated from IgG Sorb in 1% SDS. IgG Sorb was removed by sedimentation and discarded. The resulting supernatant fractions contained proteins that had previously been bound (*B*) to the IgG Sorb. Portions of each "free" and "bound" fraction were immune precipitated with ATPase (lanes *3, 4, 7, 8*) or acid phosphatase (lanes *1, 2, 5, 6*) antiserum. To facilitate quantification of acid phosphatase, the heterogeneous carbohydrate component was removed by treatment with endoglycosidase H. Samples were resolved by SDS-PAGE on a 6% gel and visualized by autoradiography.

tested with a cruder vesicle fraction which contained all membranes that sedimented in the high-speed centrifugation of a lysate. The crude vesicle fraction was exposed to the PM ATPase immune precipitation procedure followed by quantification of the bound and free fractions. If nonspecific aggregation occurred, all membranes (hence, all of the proteins present) would have bound to the IgG Sorb. Once again, equivalent amounts of acid phosphatase and ATPase were bound to the IgG Sorb complexes (not shown). Furthermore, as shown in Fig. 8, the spectrum of proteins that bound selectively to IgG Sorb (lane *5*) represented a subset of the proteins that were present in total membrane fractions. These proteins corresponded to those of purified secretory vesicles (Fig. 8, lanes *1–4*, and reference 15). Thus these experiments confirmed that the ATPase antibody recognized a unique population of secretory vesicles containing the plasma membrane protein, ATPase, and the secretory protein, acid phosphatase.

Discussion

Numerous previous studies have implicated the secretory pathway in the export of integral plasma membrane proteins. Generally, constitutive transport of proteins from the Golgi apparatus to the cell surface is rapid and the steady-state level of vesicular intermediates is low (40). For this reason, it has not been possible to identify unambiguously the vesicle(s) responsible for targeting proteins that are exported constitutively. In one case the G membrane protein of VSV-infected Hep2 cells has been visualized by immune electron microscopy in vesicles that contain the secretory proteins transferrin and albumin (41). It is difficult to quantify the extent to which this vesicle species participates in G protein export, leaving open the possibility that other pathways operate to externalize diverse cell surface molecules.

The secretory pathway in yeast operates exclusively by a constitutive process. Secretory glycoproteins and plasma membrane precursors are transported rapidly from the Golgi apparatus to the bud membrane in 60–80 nm vesicles (26). Fusion of these vesicles with the bud membrane accounts for polarized growth of the yeast cell surface (11). Although previous work established an intimate coupling between the secretion of soluble molecules and the assembly of plasma membrane proteins (27), it has not been possible until now to demonstrate that a unique vesicle species is responsible for export of both classes of molecules.

To examine the cargo in yeast secretory vesicles, we have developed an isolation procedure that optimizes for vesicle stability, yield, and resolution from other cellular membranes. First, to ensure a rich supply of material, a *sec* mutant strain known to accumulate large quantities of mature and functional vesicles was incubated at a restrictive temperature. Optimum vesicle stability was achieved when

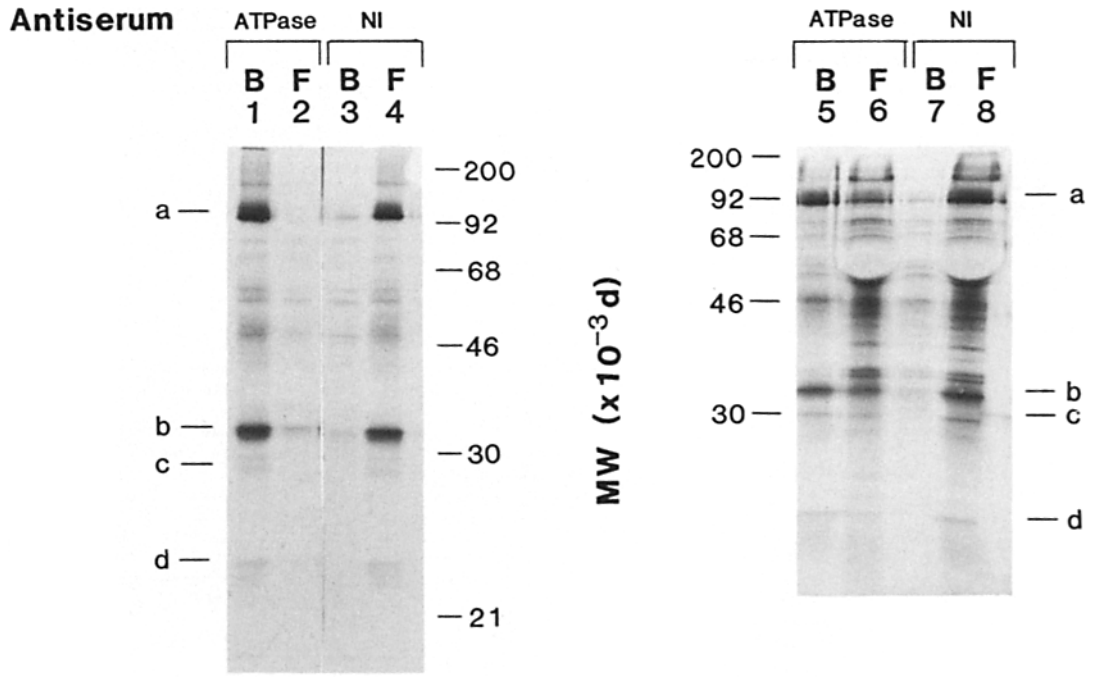

Figure 8. A discrete set of proteins coprecipitates with the ATPase in secretory vesicles. (Lanes *1–4*) Portions of the B and F fractions from the experiment in Fig. 7 were evaluated by SDS-PAGE on a 10% gel and visualized by autoradiography; *NI*, nonimmune. (Lanes *5–8*) Same as lanes *1–4*, respectively, except that aliquots of the HS pellet fraction were used instead of Percoll gradient-purified fractions.

spheroplasts rather than intact cells were allowed to accumulate vesicles. Stability of vesicles was measured during the isolation procedure by recovery of sedimentable acid phosphatase activity. Highest yield was achieved when spheroplast rupture and vesicle isolation were conducted in iso-osmotic media. These conditions proved crucial to the detection of a single vesicle species. Early experiments using what proved to be suboptimal spheroplast lysis conditions generated a mixed population of broken and intact secretory vesicles. On the Percoll gradient these forms separated into light vesicle membrane fragments and dense vesicles, giving the misleading appearance of a "vesicle" species enriched in newly synthesized plasma membrane proteins, but lacking secretory proteins. The light vesicle membrane species is nearly eliminated when conditions that preserve vesicle integrity are used.

We have considered the possibility that coincident localization of ATPase and acid phosphatase results from fusion of distinct vesicle populations in vivo or in vitro. To account for the quantitative recovery of these two classes of protein in a single vesicle species by artifact, fusion must have been both complete and specific for Golgi-derived vesicles. Two purification methods showed exactly coincident fractionation of PM ATPase and acid phosphatase, with substantial resolution from all other organelles. Furthermore, briefer periods of accumulation within spheroplasts of *secl* cells (30 min vs. 90 min at 37°C) did not alter the fractionation properties of secretory vesicles. Finally, immunoelectron microscopic examination of thin sections has shown PM ATPase and invertase (another secreted enzyme) colocalized in secretory vesicles of *secl* cells (Brada, D., and R. Schekman, manuscript submitted for publication).

In addition to the ATPase, purified secretory vesicles contain several other prominent species. Proteins a, c, and d (Fig. 8) are not released by treatment of vesicles with Na_2CO_3 (not shown), hence they represent other membrane proteins that are assembled into the bud membrane. A somewhat different procedure for the purification of yeast secretory vesicles has been described by Walworth and Novick (46). Their procedure results in vesicles with a similar spectrum of major polypeptides even though the membranes are obtained from a different *sec* mutant strain (*sec6*). It is likely that this pattern of vesicle proteins will remain constant in the membranes obtained from any of the strains in which the last step in the secretory pathway is blocked.

One important difference between our procedure and that of Walworth and Novick is the glucose concentration in the growth medium during incubation at the restrictive temperature. Our procedure employs 2% glucose; their procedure calls for 0.2% glucose, a condition that allows derepression of invertase synthesis. The higher glucose concentration is necessary to maintain a high level of ATPase synthesis. No growth conditions were found that allowed optimal synthesis of both invertase and ATPase.

Bud-localized secretory vesicles represent the major, though possibly not the only source of material for yeast surface growth. If plasma membrane proteins in the mother portion of a dividing cell are subject to some form of turnover, a replacement mode of insertion may involve distinct vesicles. Later in the division cycle, during cytokinesis, 60–80–nm vesicles localize to the region of the division septum (22). These may represent a septum-specific type of secretory vesicle, or simply reflect the dominant form of secretory vesicle made during cytokinesis. Markers for such vesicles include the chitin synthase involved in septation (chitin synthase II?, reference 4) and a chitinase that acts to resolve the

chitin ring on a mother cell from the birth scar on a daughter cell (6). A survey of these proteins in the secretory vesicle fractions would address the possibility of single or multiple vesicle types.

We are grateful to Nathan Nelson and Jeff Schatz for contributions of various antisera, and to Gordon Hammes for a sample of the PM ATPase. We thank R. Feldman and J. Rine for helpful suggestions and Peggy McCutchan-Smith for preparation of the manuscript.

This work was supported by grants from the National Institutes of Health (General Medical Sciences) and the National Science Foundation.

Received for publication 8 September 1987, and in revised form 23 October 1987.

References

1. Atkinson, K., and R. Ramirez. 1984. Secretion can proceed uncoupled from net plasma membrane expansion in inositol-starved *Saccharomyces cerevisiae*. *J. Bacteriol.* 160:80–86.
2. Bernstein, M., W. Hoffmann, G. Ammerer, and R. Schekman. 1985. Characterization of a gene product (Sec53p) required for protein assembly in the yeast endoplasmic reticulum. *J. Cell Biol.* 101:2374–2382.
3. Bordallo, C., J. Schwencke, and M. P. Suarez-Rendueles. 1984. Localization of the thermosensitive X-prolyl dipeptidyl aminopeptidase in the vacuolar membrane of *Saccharomyces cerevisiae*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 173:199–203.
4. Bulawa, C. E., M. Slater, E. Cabib, J. Au-Young, A. Sburlati, W. L. Adair, and P. W. Robbins. 1986. The *Saccharomyces cerevisiae* structural gene for chitin synthase is not required for chitin synthesis *in vivo*. *Cell.* 46:213–225.
5. Deshaies, R., and R. Schekman. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* 105:633–645.
6. Elango, N., J. U. Correa, and E. Cabib. 1982. Secretory character of yeast chitinase. *J. Biol. Chem.* 257:1398–1400.
7. Esmon, B., P. Novick, and R. Schekman. 1981. Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. *Cell.* 25:451–460.
8. Esmon, P. C. 1985. Molecular and organelle intermediates in the secretory process. Ph.D. thesis. University of California, Berkeley, CA. 124 pp.
9. Feldman, R., M. Bernstein, and R. Schekman. 1987. Product of *SEC53* is required for folding and glycosylation of secretory proteins in the lumen of the yeast endoplasmic reticulum. *J. Biol. Chem.* 262:9332–9339.
10. Ferro-Novick, S., P. Novick, C. Field, and R. Schekman. 1984. Yeast secretory mutants that block the formation of active cell surface enzymes. *J. Cell Biol.* 98:35–43.
11. Field, C., and R. Schekman. 1980. Localized secretion of acid phosphatase reflects the pattern of cell surface growth in *Saccharomyces cerevisiae*. *J. Cell Biol.* 86:123–128.
12. Fuller, S. D., R. Bravo, and K. Simons. 1985. An enzymatic assay reveals that proteins destined for the apical and basolateral domains of an epithelial cell line share the same late Golgi compartments. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:297–307.
13. Gumbiner, B., and R. B. Kelly. 1982. Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. *Cell.* 28:51–59.
14. Hansen, W. 1985. Intracellular transport of yeast secretory and plasma membrane proteins. Ph.D. thesis. University of California, Berkeley. 183 pp.
15. Holcomb, C. L., T. Etcheverry, and R. Schekman. 1987. Isolation of secretory vesicles from *Saccharomyces cerevisiae*. *Anal. Biochem.* 166:328–334.
16. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.).* 195:495–496.
17. Julius, D., L. Blair, A. Brake, G. Sprague, and J. Thorner. 1983. Yeast α-factor is processed from a larger precursor polypeptide: the essential role of a membrane-bound dipeptidyl aminopeptidase. *Cell.* 32:839–852.
18. Kelly, R. B. 1985. Pathways of protein secretion in eukaryotes. *Science (Wash. DC).* 230:25–32.
19. Koland, J., and G. G. Hammes. 1986. Steady-state kinetic studies of purified yeast plasma membrane proton-translocating ATPase. *J. Biol. Chem.* 261:5936–5942.
20. Kubota, S. Y., Y. Yoshida, H. Tumaoka, and H. Furumichi. 1977. Studies on the microsomal electron transport system of anaerobically-grown yeast. V. Purification and characterization of NADPH-cytochrome *c* reductase. *J. Biol. Chem.* 81:197–206.
21. Lodish, H. F., W. A. Braell, A. L. Schwartz, G. J. M. Strous, and A. Zilberstein. 1981. Synthesis and assembly of membrane and organelle proteins. *Int. Rev. Cytol.* (Suppl. 12):247–309.
22. Matile, P. H., H. Moor, and C. F. Rabinow. 1969. Yeast Cytology. *In* The Yeasts. Vol. 1. A. H. Rose and J. S. Harrison, editors. Academic Press, New York. 220–302.
23. Moore, H.-P., B. Gumbiner, and R. B. Kelly. 1983. A subclass of proteins and sulfated macromolecules secreted by AtT-20 (mouse pituitary tumor) cells is sorted with adrenocorticotropin into dense secretory granules. *J. Cell Biol.* 97:810–817.
24. Moore, H.-P., M. Walker, F. Lee, and R. B. Kelly. 1983. Expression a human proinsulin cDNA in a mouse ACTH secretory cell. Intracellular storage, proteolytic processing and secretion on stimulation. *Cell.* 35: 531–538.
25. Nakajima, T., and C. Ballou. 1975. Yeast manno-protein biosynthesis: solubilization and selective assay of four mannosyl-transferases. *Proc. Natl. Acad. Sci. USA.* 72:3912–3916.
26. Novick, P., and R. Schekman. 1979. Secretion and cell surface growth are blocked in a temperature sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 76:1858–1862.
27. Novick, P., and R. Schekman. 1983. Export of major cell surface proteins is blocked in yeast secretory mutants. *J. Cell Biol.* 96:541–547.
28. Novick, P., S. Ferro, and R. Schekman. 1981. Order of events in the yeast secretory pathway. *Cell.* 25:461–469.
29. Novick, P., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell.* 21:205–215.
30. Palade, G. 1975. Intracellular aspects of the process of protein synthesis. *Science (Wash. DC).* 189:347–358.
31. Pfeffer, S., and R. Kelly. 1985. The subpopulation of brain coated vesicles that carries synaptic vesicle proteins contains two unique polypeptides. *Cell.* 40:949–957.
32. Rindler, M. J., I. E. Ivanov, H. Plesken, E. Rodriguez-Boulan, and D. D. Sabatini. 1984. Viral glycoproteins destined for apical and basolateral plasma membranes traverse the same Golgi apparatus during their intracellular transport in double-infected Madin-Darby canine kidney cells. *J. Cell Biol.* 98:1304–1319.
33. Rodriguez-Bouland, E. J., and D. D. Sabatini. 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for the study of epithelial cell polarity. *Proc. Natl. Acad. Sci. USA.* 5:5071–5075.
34. Schauer, I., S. Emr, C. Grass, and R. Schekman. 1985. Invertase signal and mature sequence substitutions that delay intercompartmental transport of active enzyme. *J. Cell Biol.* 100:1664–1675.
35. Scott, J., and R. Schekman. 1980. Lyticase: endoglucanase and protese activities that act together in yeast cell lysis. *J. Bacteriol.* 142:414–423.
36. Serrano, R. 1984. Plasma membrane ATPase of fungi and plants as a novel type of proton pump. *Curr. Top. Cell. Regul.* 23:87–126.
37. Serrano, R., M. C. Kielland-Brandt, and G. R. Fink. 1986. Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+, K^+), K^+- and Ca^{2+}-ATPases. *Nature (Lond.).* 319:689–693.
38. Simons, K., and S. D. Fuller. 1985. Cell surface polarity in epithelia. *Annu. Rev. Cell Biol.* 1:243–288.
39. Stevens, T., B. Esmon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* 30:439–448.
40. Strous, G., and H. Lodish. 1980. Intracellular transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. *Cell.* 22:709–717.
41. Strous, G. J. A. M., R. Willemsen, P. VanKerkhof, J. W. Slot, W. Geuze, and H. Lodish. 1983. Vesicular stomatitis virus glycoprotein, albumin, and transferrin are transported to the cell surface via the same Golgi vesicles. *J. Cell Biol.* 97:1815–1822.
42. Toh-e, A., Y. Ueda, S.-I. Kakimoto, and Y. Oshima. 1973. Isolation and characterization of acid phosphatase mutants in *Saccharomyces cerevisiae*. *J. Bacteriol.* 113:727–738.
43. Tschopp, J., P. C. Esmon, and R. Schekman. 1984. Defective plasma membrane assembly in yeast secretory mutants. *J. Bacteriol.* 160: 966–970.
44. van Rijn, H. J. M., P. Boer, and E. P. Steyn-Parve. 1972. Biosynthesis of acid phosphatase of baker's yeast. Factors influencing its production by protoplasts and characterization of the secreted enzyme. *Biochim. Biophys. Acta.* 268:431–441.
45. Vincent, R., and D. Nadeau. 1983. A micromethod for the quantitation of cellular proteins in Percoll with the Coomassie brilliant blue dye-binding assay. *Anal. Biochem.* 135:355–362.
46. Walworth, N. C., and P. N. Novick. 1987. Purification and characterization of constitutive secretory vesicles from yeast. *J. Cell Biol.* 105:163–174.