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Defective Plasma Membrane Assembly in Yeast Secretory Mutants

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Yeast mutants that are conditionally blocked at distinctive steps in secretion and export of cell surface proteins have been used to monitor assembly of integral plasma membrane proteins. Mutants blocked in transport from the endoplasmic reticulum (*sec18*), from the Golgi body (*sec7* and *sec14*), and in transport of secretory vesicles (*sec1*) show dramatically reduced assembly of galactose and arginine permease activities. Simultaneous induction of galactose permease and α-galactosidase (a secreted glycoprotein) in *sec* mutant cells at the nonpermissive temperature (37°C) shows that both activities accumulate and can be exported coordinately when cells are returned to the permissive temperature (24°C) in the presence or absence of cycloheximide. Plasma membrane fractions isolated from *sec* mutant cells radiolabeled at 37°C have been analyzed by two-dimensional sodium dodecyl sulfate-gel electrophoresis. Although most of the major protein species seen in plasma membranes from wild-type cells are not efficiently localized in *sec18* or *sec7*, several of these proteins appear in plasma membranes from *sec1* cells. These results may be explained by contamination of plasma membrane fractions with precursor vesicles that accumulate in *sec1* cells. Alternatively, some proteins may branch off during transport along the secretory pathway and be inserted into the plasma membrane by a different mechanism.

A series of membrane-bounded structures mediates the transfer of exported proteins from their site of synthesis at the rough endoplasmic reticulum to their site of discharge at the plasma membrane. In yeast cells, previous work from our laboratory suggests that plasma membrane proteins and secretory proteins share a common pathway for secretion (3, 4, 10–12). It is not clear, however, whether plasma membrane proteins are transferred through the same series of membrane-bounded structures as secretory proteins or whether there are branch points along that pathway as is found for vacuolar carboxypeptidase Y (16).

The availability of conditional mutants blocked in secretion and cell surface growth provides a means to study this possibility. The *sec* mutants define three stages along a linear pathway (10), movement from the rough endoplasmic reticulum to the Golgi body, transport through the Golgi body, and transport of vesicles to the cell surface. In *sec* mutant cells, transport is interrupted at a nonpermissive temperature (37°C), and accumulated material is discharged on return to the permissive temperature (25°C).

In this report we describe the effects of genetic blocks in the secretory pathway on incorporation of proteins into the plasma membrane. We chose a rough endoplasmic reticulum-accumlating mutant (*sec18*), Golgi body-accumulating mutants (*sec7 sec14*), and a vesicle-accumulating mutant (*sec1*). To determine whether the secretion pathway and plasma membrane assembly are colinear, we compared the export of galactose permease and α-galactosidase, markers of the plasma membrane and periplasm, respectively. We also evaluated the transport of unidentified polypeptides enriched in plasma membranes isolated from wild-type and *sec* mutant cells.

MATERIALS AND METHODS

Saccharomyces cerevisiae strain X2180-1A was obtained from the Yeast Genetics Stock Center, Berkeley, Calif. *sec* mutant strains were described previously (11). 108-3Cα (*MEL1*) was a gift from Bruce G. Adams, University of Hawaii, Honolulu, and haploid *sec* Gal^+ *MEL1* mutants were constructed by standard genetic techniques and were monitored for temperature-sensitive growth (*sec* phenotype). In the galactose permease experiments, cells were grown in YP medium (1% yeast extract, 2% Bacto-Peptone) with the indicated carbon source. In other experiments cells were grown in the minimal medium described by Wickerham (18); chloride salts replaced all sulfate salts, and the carbon source was 2% glucose supplemented with 0.05 mM $(NH_4)_2SO_4$. Cultures were grown in flasks with agitation and initiated with cells from stationary stock cultures at an optical density measured at 600 nm (OD_{600}) of 30 to 40. The absorbance of all suspensions was measured in a 1-cm quartz cuvette at 600 nm in a Zeiss PM QII spectrophotometer; 1 OD_{600} U corresponded to 10^7 cells per ml or 0.15 mg (dry weight).

Concanavalin A, ATP (grade I, vanadate-free), and *p*-nitrophenyl-α-D-galactopyranoside were obtained from Sigma Chemical Co., St. Louis, Mo.; $H_2[^{35}S]O_4$, $[^3H]$D-galactose, [*G*-^{3}H]arginine, Na$[^{125}I]$, and En^{3}Hance were obtained from New England Nuclear Corp., Boston, Mass. Scint-A was obtained from Packard Instrument Co., Inc., Downers Grove, Ill. Renografin-76 was obtained from E. R. Squibb & Sons, Princeton, N.J. Nonidet P-40 was obtained from BDH Chemicals Ltd., Poole, England. All other chemicals used were of reagent-grade purity.

Galactose permease assays. Galactose permease assays were performed by the method of Shou-Chang et al. (14), with the following modifications. Cells were grown at 24°C in YP medium with 2% ethanol as a carbon source to an OD_{600} of 5 to 10. Cells were centrifuged, suspended in new YP medium containing 0.5% galactose and 0.05% glucose at an OD_{600} of 5 to 10, and divided into two parts. In one culture, incubation was continued at 24°C, and the other one was shifted to 37°C for 2 h. To one half of the 37°C culture, cycloheximide was added to a final concentration of 0.2 mg/ml, using a stock solution at 10 mg/ml. Incubation was continued at 24°C for 2 h. Cells (10 OD_{600} U) were withdrawn from the culture medium at the indicated times,

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centrifuged, and washed once with 1 ml of ice-cold 20 mM sodium azide. The cell pellet was kept on ice until assayed. Assay mixture (0.1 ml) containing 2×10^4 to 3×10^4 cpm of [^{3}H]D-galactose in 0.25 mM D-galactose was added to the washed cells. The cell suspension was shaken vigorously for 5 min in a 37°C waterbath, cooled on ice immediately, filtered through a membrane filter (HA, 0.45 μm, pore size; Millipore Corp., Bedford, Mass.), and washed with 1 ml of cold water. Filters were air dried and mixed with 3 ml of scintillation fluid, and radioactivity was measured in a Searle Delta 300 scintillation counter. Uptake was linear up to 10 min, and 1 unit represents uptake of 1 μmol of D-galactose per min at 37°C.

Arginine permease assays. Arginine permease was assayed by the method of Grenson et al. (5), with the following modifications. Cells were grown in the minimal medium described by Wickerham (18), containing 2% glucose and 5 mM arginine, to early log phase. Cells were harvested, washed, suspended in arginine-free medium, and incubated at 24 or 37°C. Assay mixtures contained 1 OD_{600} U of washed cells and 50 μM [G-^{3}H]arginine (8×10^4 cpm) in a final volume of 1 ml. Reaction mixtures were incubated for 0 to 1 min at 37°C, and reactions were terminated by adding 4 ml of ice-cold 5 mM arginine; mixtures were then filtered through Millipore filters (HA, 0.45 μm pore size). The filters were washed three times with 4 ml of ice-cold water, dried, and counted in 3 ml of scintillation fluid. One unit of arginine permease activity represents 1 nmol of arginine taken up per min at 37°C.

Determination of α-galactosidase. The α-galactosidase assay was adopted from the method of Kew and Douglas (7). Cells (1 OD_{600} U) were withdrawn from the cultures at the indicated times, centrifuged, and washed with 1 ml of cold water. The assay mixture (0.1 ml) containing 1.5 mg of p-nitrophenyl-α-D-galactopyranoside per ml in 31 mM citric acid (buffered at pH 4 with 39 mM KH_2PO_4) was added to the cell pellet. The cell suspension was incubated at 37°C for 15 to 30 min with gentle agitation, and the reaction was stopped by the addition of 0.9 ml of 0.1 M Na_2CO_3. Cells were centrifuged, and the absorbance of the supernatant solution was measured at 400 nm. One unit of α-galactosidase represents 1 nmol of p-nitrophenol released per min at 37°C.

Other procedures. For plasma membrane isolation, cells were grown overnight at 24°C to an OD_{600} of 1 to 2 in sulfate-free minimal medium (10, 18) supplemented with 0.05 mM $(NH_4)_2SO_4$ and 2% glucose. After centrifugation, cells were suspended in fresh medium (500 ml) at an OD_{600} of 1.2 to 1.5. After 15 min at 37°C, 1 to 2 mCi of carrier-free $H_2[^{35}S]O_4$ was added, and incubation continued for 1 h. Labeling was terminated by cooling the culture to 0°C, and NaN_3 was added to 40 mM (final concentration). Cells were collected by centrifugation, and plasma membrane fractions were obtained as described previously (17). Plasma membrane pellet fractions were suspended in 1 ml of lysis buffer (10 mM Tris-hydrochloride [pH 7.5], 5 mM $MgSO_4$). Between 0.2 and 0.4 mg (1×10^6 to 2×10^6 cpm) of membrane protein (gradient peaks B or C) was used for two-dimensional gel electrophoresis. Radiolabel uptake in *sec* mutant cells at 37°C was between 40 and 60% of the wild-type level. *sec* mutant plasma membrane fractions had a specific radioactivity (counts per minute per milligram of membrane protein) two- to fourfold lower than that of wild-type plasma membrane fractions.

Two-dimensional gel electrophoresis was performed as described by O'Farell (13). Membrane protein was solubilized for 3 min in a water bath at 90°C. Insoluble aggregates were removed by centrifugation at 30,000 × g for 30 min. After electrophoresis in the second dimension, the 10% polyacrylamide gel was stained with Coomassie blue, destained, treated with En^{3}Hance, dried, and exposed to Kodak X-Omat R film at −70°C. Molecular weight standards were phosphorylase a (M_r = 84,000), bovine serum albumin (M_r = 68,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), and trypsin inhibitor (M_r = 20,000).

Protein was estimated by the method of Lowry et al. (9). Bovine serum albumin was used as a standard.

ATPase assays were performed with unlabeled cells as described earlier (17).

RESULTS

Export of galactose permease and α-galactosidase blocked reversibly. In galactose-fermenting strains of *S. cerevisiae*, galactose permease, a component of the galactose transport system, is induced by exogenous galactose (7, 14). When wild-type cells are transferred from medium containing a nonfermentable carbon source, such as ethanol, to medium containing galactose, a large increase in galactose permease activity occurs. A similar induction was observed in *sec* mutant cells at the permissive growth temperature (24°C) (Fig. 1A). However, all *sec* mutants failed to show this activity during a 2-h induction at 37°C. Upon return to the permissive temperature in the presence or absence of cycloheximide, galactose permease activity was detected in the *sec* mutants.

Since galactose and melibiose utilization is coregulated in melibiose-fermenting strains, Gal$^+$ *MEL1* cells are simultaneously induced for galactose permease and the periplasmic α-galactosidase (7). The level of secreted α-galactosidase increased 10-fold in cells which were transferred into the galactose-containing medium. This increase was also blocked in *sec* mutants at 37°C, but not in those at 24°C (Fig. 1B). Upon return to 24°C, in the presence of cycloheximide, all mutants secreted a large fraction of accumulated active enzyme. *sec* mutants from other complementation groups showed the same phenotype with respect to galactose permease incorporation and α-galactosidase secretion (data not shown).

Arginine permease incorporation blocked. Arginine permease synthesis is induced when cells growing in a medium containing 5 mM arginine are transferred to an arginine-free medium (5). When X2180-1A and *sec1* cells were transferred to 37°C in medium without arginine, permease activity was lost during the first 30 min. This appeared to be due to inactivation of the existing pool of cell-surface permease. Thereafter, permease incorporation continued in wild-type cells but not in *sec1* mutant cells (Fig. 2). All *sec* mutants from other complementation groups produced normal levels of permease activity during a 2-h period of induction at 24°C but showed significantly lower incorporation at 37°C (data not shown).

Proteins in *sec* mutant plasma membrane fractions. To investigate the assembly of a wider range of plasma membrane proteins, wild-type and *sec* mutant cells were radiolabeled at 37°C and plasma membrane fractions were purified by a procedure that removes other major intracellular membranes (17). Purification and yield were assessed by monitoring vanadate-sensitive ATPase activity and ^{125}I label. During purification, plasma membrane fractions from wild-type and different *sec* mutant cells showed similar differential and density sedimentation characteristics. The final purification step, Renografin equilibrium centrifugation, pro-

duced three membrane fractions (A, B, and C) as described before (17). Fractions B and C contained 70 to 80% of the vanadate-sensitive ATPase applied to the gradient and 15 to 20% of the activity found in the original extracts (Table 1). These two fractions were evaluated by two-dimensional sodium dodecyl sulfate-gel electrophoresis of detergent-solubilized proteins, using conditions described by O'Farell (13). To normalize differences in labeling efficiency among the four strains used, equal amounts of ^{35}S-radioactivity were applied to all gels. Figure 3 shows autoradiograms of eight two-dimensional gels of proteins from X2180-1A, *sec1*, *sec7*, and *sec18* plasma membrane fractions. Six major species or groups are indicated which illustrate the pattern that emerged in this investigation. The major proteins have isoelectric points of 5 to 6.5 and molecular weights of 40,000 to 100,000. The most dramatic reduction in assembly was seen in *sec18*. *sec7* membranes contained only moderately reduced levels of species 4 and 5, whereas all six proteins appeared nearly normal in *sec1* membranes. Treatment of membrane fractions with high levels of salt (1.7 M NaCl) or alkali (0.1 N NaOH), conditions which elute peripheral proteins from other membranes, had no effect on the appearance of radiolabeled proteins in the wild-type or mutant plasma membranes.

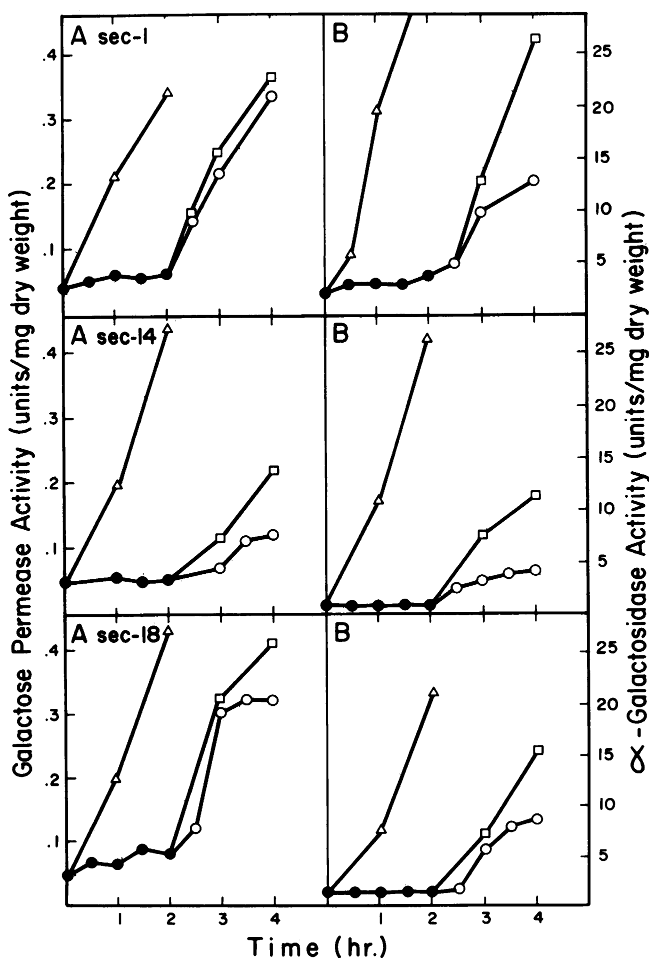

FIG. 1. Galactose permease (A) and α-galactosidase (B) accumulation in *sec1*, *sec14*, and *sec18*. Cells were grown at 24°C in YP medium 2% ethanol. At time zero, the cells were transferred to YP medium–0.5% galactose–0.05% glucose and shifted to 24°C (△) or 37°C (●). At 2 h the 37°C culture was shifted back to 24°C without (□) or with (○) cycloheximide (0.2 mg/ml). Samples were withdrawn, chilled to 0°C, and washed in 20 mM sodium azide. Galactose permease and secreted α-galactosidase were measured.

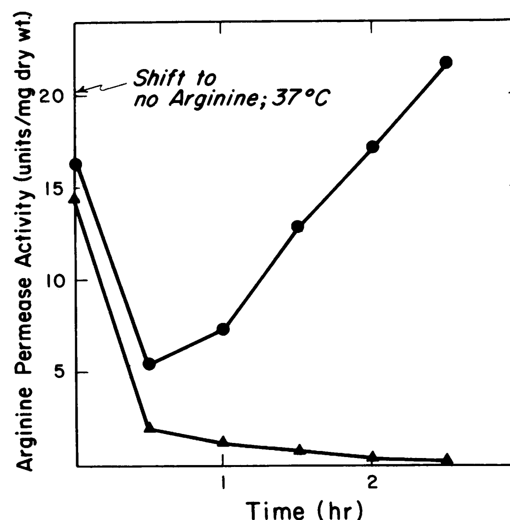

FIG. 2. Incorporation of arginine permease in X2180-1A (●) and HMSF1 *sec1* (▲). Cells were grown at 24°C in minimal medium containing 5 mM arginine and 2% glucose. At time zero, the cells were transferred to arginine-free minimal medium, and incubation was continued as indicated in the figure. Samples were withdrawn, chilled to 0°C, centrifuged, and resuspended in arginine-free medium. Activity was then measured.

With the experimental conditions for protein solubilization described by O'Farell (13), only 25 to 35% of the radioactivity in the B and C fractions was released in a nonsedimentable form. Sodium dodecyl sulfate solubilization, as described by Ames and Nikaido (1); was more efficient (90 to 95% solubilization) but produced poorly focused protein species that were difficult to interpret.

DISCUSSION

The behavior of the yeast secretory mutants suggests a tight coupling of secretion and cell-surface assembly. Mutants blocked at steps along the entire pathway accumulate precursors of secreted enzymes and membrane-bounded organelles (11). Previous efforts to strengthen this connection have relied on measurements of secreted enzymes (invertase and acid phosphatase) and permease activities (sulfate permease) that are induced under different metabolic conditions (11) and on surface labeling of major polypeptides located in the periplasm or on the exterior of the plasma membrane (12).

In this report we have extended our studies on the coordination of secretion and plasma membrane assembly.

TABLE 1. Distribution of membranes from X2180-1A, *sec1*, *sec7*, and *sec18* on Renografin gradients

Gradient peak	Density (g/cm^3)	ATPase[a] (%)	Protein[a] (%)	^{125}I[b] (%)
A	<1.12	<5	<10	<1
B	1.13–1.15	70–80	30–40	20–30
C	1.17	15–25	50–60	70–80

[a] Values for vanadate-sensitive ATPase and protein represent the range from three preparations of each of four strains.
[b] Values from X2180-1A and *sec1* only (see reference 17).

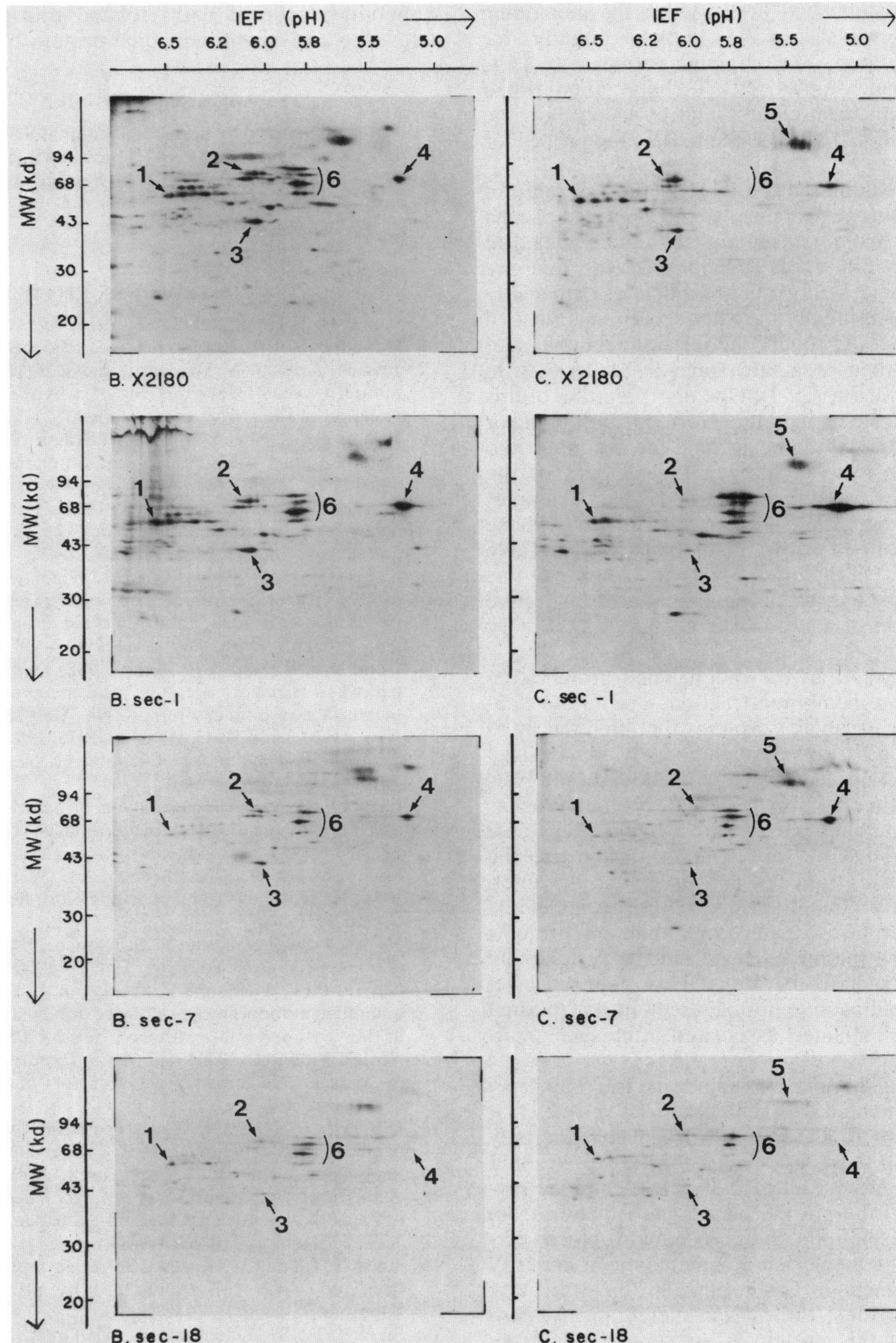

FIG. 3. Comparative two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{35}S-labeled, solubilized B and C membrane fractions from Renografin gradients. Solubilized membranes (2.5×10^5 cpm of ^{35}S) were loaded on each gel. Exposure time was 10 days. The horizontal arrow at the top of the figure indicates the direction of the isoelectric focusing and the vertical arrow indicates the direction of the sodium dodecyl sulfate migration. Six major protein species are indicated.

Two additional permease activities and another secreted enzyme are now shown to rely on the secretory pathway for export. Because of coordinate induction of melibiose and galactose utilization in yeast cells, simultaneous synthesis of genetically well-defined secreted (α-galactosidase, *MEL1*) and plasma membrane (galactose permease, *GAL2*) proteins allowed monitoring of export in a single metabolic condition. Both activities are accumulated internally in *sec* mutant cells at 37°C and exported when cells are returned to 24°C in the presence or absence of cycloheximide. Similarly, export of arginine permease is blocked at 37°C in all of the *sec* mutants. Although the transport systems may contain only one membrane component, many bacterial transport reactions require several proteins. The failure to export permease activity could be due to accumulation of a periplasmic binding protein, rather than an integral membrane protein.

This point can be established firmly when the membrane component of a permease can be followed directly, for example, with an antibody. The arginine permease (*CAN1*) (2) and galactose permease (*GAL2*; J. Tschopp, unpublished data) genes have both been cloned, and by using the gene fusion approach, it should be possible to develop probes for the gene products.

In an attempt to examine more directly the assembly of integral plasma membrane proteins, we have purified plasma membrane fractions from *sec* mutant cells radiolabeled at the restrictive temperature. The major proteins that are solubilized by nonionic detergents are much less efficiently assembled in the plasma membrane when secretion is blocked at an early stage (*sec18*; rough endoplasmic reticulum to Golgi body block) than at a late stage (*sec1*; vesicle to plasma membrane block) (Fig. 3). One interpretation of this result is that these proteins may be diverted from the major pathway at some late stage and become incorporated into the plasma membrane in a pathway independent of the late-acting *SEC* gene products. If true, these examples would contrast with membrane proteins and functions whose assembly is deficient in all of the *sec* mutants (Fig. 1, Table 1, and reference 12). On the other hand, an example of this divergence is documented in the regulated and constitutive pathways of secretion of adrenocorticotropin in a pituitary cell line (6).

Another possibility is that the radiolabeled proteins detected in *sec1* plasma membrane fractions are due to contaminating soluble or organellar proteins that bind tightly to membrane components. For example, newly synthesized hydrolases could be released from the vacuole during lysis and redistribute to other membranes. Since proteins such as carboxypeptidase Y are activated in the vacuole after transport from the Golgi body (16), only mutants blocked after the Golgi step (which do not affect transport to the vacuole), such as *sec1*, would produce labeled, mature forms of the major vacuolar hydrolases. Such contaminating proteins should be eluted by treatments that remove peripherally associated proteins, such as 0.1 N NaOH or high salt level (15). However, the radioactive proteins seen in *sec1* plasma membrane fractions remained associated after such treatments.

A final possibility is that plasma membrane fractions from *sec1* cells are contaminated with membranes of vesicles that are blocked in transit to the cell surface. Thus far, only secreted enzymes can serve as markers for the vesicles that accumulate in *sec1*. Almost all of the vesicle-bounded invertase is removed during plasma membrane isolation from *sec1* cells (17). The isolation conditions could rupture secretory vesicles, and the membrane fragments might copurify with plasma membranes. In this regard, we have recently produced antibody against the plasma membrane ATPase and have found that this protein is not assembled in the plasma membrane in *sec* mutant cells at 37°C (W. Hansen, J. Tschopp, and R. Schekman, unpublished data). Thus, organelles that mediate ATPase transport are resolved from plasma membranes by our isolation procedure.

Future work on the pathway(s) of plasma membrane assembly in yeast cells will require specific probes, such as antibodies, coupled with isolated transport organelles to allow the analysis of individual protein species.

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