Export of Major Cell Surface Proteins Is Blocked in Yeast Secretory Mutants

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ABSTRACT The transport of newly synthesized proteins to the yeast cell surface has been analyzed by a modification of the technique developed by Kaplan et al. (Kaplan, G., C. Unkeless, and Z. A. Cohn, 1979, *Proc. Natl. Acad. Sci. USA*, 76:3824–3828). Cells metabolically labeled with $^{35}SO_4^{2-}$ are treated with trinitrobenzenesulfonic acid (TNBS) at 0°C under conditions where cell-surface proteins are tagged with trinitrophenol (TNP) but cytoplasmic proteins are not. After fractionation of cells into cell wall, membrane and cytoplasmic samples, and solubilization with SDS, the tagged proteins are immunoprecipitated with anti-TNP antibody and fixed *Staphylococcus aureus* cells. Analysis of the precipitates by SDS gel electrophoresis and fluorography reveals four major protein species in the cell wall (S_1-S_4), seven species in the membrane fraction (M_1-M_7), and no tagged proteins in the cytoplasmic fraction.

Temperature-sensitive mutants defective in secretion of invertase and acid phosphatase (*sec* mutants; Novick, P., C. Field, and R. Schekman, 1980, *Cell*, 21:204–215) are also defective in transport of the 11 major cell surface proteins at the nonpermissive temperature (37°C). Export of accumulated proteins is restored in an energy-dependent fashion when *sec1* cells are returned to a permissive temperature (24°C). In wild-type cells the transit time for different surface proteins varies from less than 8 min to about 30 min. The asynchrony is developed at an early stage in the secretory pathway.

All of the major cell wall proteins and many of the externally exposed plasma membrane proteins bind to concanavalin A. Inhibition of asparagine-linked glycosylation with tunicamycin does not prevent transport of several surface proteins.

Several independent lines of evidence suggest that eukaryotic cell surface proteins are transported via the secretory process. The most extensive evidence concerns the biosynthesis and export of viral membrane glycoproteins, among which the VSV G protein is best characterized. Biochemical, cell fractionation, immunoelectron microscopy, and genetic experiments all support a pathway in which VSV G protein is synthesized by endoplasmic-reticulum-associated ribosomes and transported to the plasma membrane via the Golgi-body and vesicles (1, 2, 3, 4). Less extensive but similar sorts of experiments support this mode of export for cellular plasma membrane proteins (5, 6).

Recent reports have suggested specialization of the Golgi apparatus to account for movement of distinct groups of exported proteins. Monensin, an ionophore that blocks transport of most proteins within the Golgi body (7), causes some glycoproteins to arrest before and some after, conversion of *N*-glycosidically linked oligosaccharides from the early high-mannose form to the mature complex form (8, 9). In one extreme case, influenza virus, which contains glycoproteins with complex oligosaccharides, buds normally from cells treated with monensin (10). Similarly, Gumbiner and Kelly (11) have proposed a branch point in the Golgi body to account for the production of ACTH granules in a pituitary cell line that secretes hormone in a regulated fashion, while exporting an endogenous viral glycoprotein constitutively.

We have developed an alternate approach to the study of secretion and cell surface assembly. The cellular components that execute the secretory process in yeast have been identified genetically by the isolation of temperature-sensitive lethal mutants that block secretion (12, 13). Secretory mutants (*sec*) accumulate glycoproteins inside one of three distinct organ-

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 FEBRUARY 1983 541-547

elles: endoplasmic reticulum (ER), Golgi bodies, or secretory vesicles. Analysis of double *sec* mutants has shown that the blocks define a linear pathway along which glycoprotein oligosaccharides are assembled much as in mammalian cells (14, 15). The *sec* mutants are also defective in bud growth and in the export of at least four permease activities (SO_4^{2-}, arginine, galactose, and proline-specific permease). These properties, together with the substantial accumulation of membrane within *sec* mutant cells, led us to propose an essential contribution of the secretory process to plasma membrane assembly (13).

Kaplan, Unkeless, and Cohn (16) devised a procedure for the analysis of plasma membrane synthesis and turnover in macrophages that we have adapted to evaluate yeast cell surface assembly. This procedure has now allowed a more specific appraisal of transport to the cell wall (secreted proteins) and to the external surface of the plasma membrane. The results presented here suggest that in yeast the *sec* gene products are responsible for export of the major cell surface proteins.

MATERIALS AND METHODS

Strains and Media: The *Saccharomyces cerevisiae* strain X2180-1A was from the Yeast Genetic Stock Center (Berkeley, CA). Mutant strains HMSF1 (*sec1*-1), SF294-2B (*sec7*-1), HMSF176 (*sec18*-1), and HMSF136 (*sec6*-4) were derived from X2180-1A (12, 13).

Wickerham's minimal medium (17) was used with the following modification: the ammonium sulfate concentration was lowered to 50 μM unless otherwise indicated, ammonium chloride was used as the nitrogen source, and the potassium phosphate concentration was raised to 20 mM to increase the buffering capacity. The carbon source was 2% glucose unless otherwise indicated. Liquid cultures were grown at 25°C in flasks or tubes with agitation, and the experiments were initiated with exponentially growing cells at an A_{600} of 3.0–4.5. The absorbance of cell suspensions was measured in a 1 cm quartz cuvette at 600 nm in a Zeiss PM QII spectrophotometer; 1 A_{600} unit corresponds to 0.15 mg dry weight.

Reagents: Reagents were obtained as indicated: trinitrobenzenesulfonic acid, cycloheximide, tunicamycin, and concanavalin A coupled to Sepharose 4-B (8 mg protein/ml gel) from Sigma Chemical Co., St. Louis, MO; carrier-free $H_2{}^{35}SO_4$ from Amersham, Arlington Heights, IL; Na ^{125}I and Enhance from New England Nuclear, Boston, MA; Iodo-gen from Pierce Chemical Co., Rockford, IL; fixed *Staphylococcus aureus* (IgG Sorb) from The Enzyme Center, Boston, MA. Antiserum prepared against TNP-keyhole limpet hemocyanin was generously provided by Dr. Ann Good. Anti-TNP IgG was purified by adsorption to and elution from DNP-bovine serum albumin coupled to Sepharose 4-B (18). Lyticase, an enzyme preparation containing both a β1-3 endoglucanase and a protease which act synergistically to hydrolyze yeast cell walls (19), was prepared by Irene Schauer; purified glucanase (Fraction III lyticase), free of protease, was prepared by Janet Scott.

Evaluation of Cell Surface Proteins

IODINATION: Cells (1–3 A_{600} U) grown in low-sulfate minimal medium were sedimented in a clinical centrifuge, washed twice with 0.15 M NaCl, 20 mM sodium phosphate (pH 7.5), resuspended in 1 ml of the same buffer, and gently transferred to a tube in which 0.1 mg of Iodogen had been dried down from a chloroform solution (1 mg/ml) (20). Na ^{125}I (100 μCi) was added and the iodination reaction was allowed to proceed for 15 min at 0°C. The cell suspension was removed and cells were washed three times with buffer. Cells were lysed by the techniques described in the legend to Fig. 1. Extracts diluted in sample buffer (2% SDS, 2% β-mercaptoethanol, 71 mM Tris·Cl [pH 6.8], 14% glycerol) were heated in a water bath at 94°C for 2 min. Aliquots (30 μl) were applied to 10% polyacrylamide slab gels (21) and electrophoresed at 30 mA for 2 h. Gels were dried and exposed on prefogged Kodak X-Omat R film with a Dupont Cronex intensifying screen. Molecular weight markers were: RNA polymerase, 165 and 155 kilodaltons (kdal); phosphorylase, 94 kdal; bovine serum albumin, 68 kdal; ovalbumin, 43 kdal; carbonic anhydrase, 30 kdal; and trypsin inhibitor 21.5 kdal.

TNBS TAGGING: Cells (0.67 A_{600} U) grown overnight in low-sulfate minimal medium were sedimented for 1 min in a clinical centrifuge and resuspended in 0.25 ml of fresh medium. The culture was incubated at 25° or 37°C and radiolabeled (where indicated) with 100–200 μCi of $^{35}SO_4^{2-}$ for various times. Cells were sedimented, washed once with 0.3 M NaCl, 0.25 mM $CaCl_2$, 50 mM sodium phosphate (pH 7.5), resuspended in 10 mM TNBS, 0.25 mM $CaCl_2$, 0.1 M sodium bicarbonate (pH 9.2), and incubated at 0°C for 30 min. Tagged cells were sedimented and washed twice at 4°C with 0.3 M NaCl, 20 mM sodium phosphate (pH 7.5). An aliquot was placed on a glass fiber disc, dried, and $^{35}SO_4^{2-}$ incorporation measured in a Searle Delta 300 liquid scintillation counter. Treated cells were diluted with untreated wild-type cells (10 A_{600} U), and the mixture was sedimented and resuspended in 100 μl of spheroplasting medium (1.2 M NaCl, 50 mM sodium phosphate (pH 7.5), 50 mM β-mercaptoethanol) with 120 U of purified lytic glucanase (19). After incubation for 50 min at 30°C, the resulting spheroplasts were chilled and sedimented at 3,000 *g* for 5 min. The supernatant fraction was transferred to a new tube, and the spheroplasts in the pellet fraction were lysed by resuspending in 100 μl of 50 mM sodium phosphate (pH 7.5), 50 mM β-mercaptoethanol. The resulting membranes were sedimented at 17,300 *g* for 10 min; the supernatant fraction which contained cytoplasmic proteins was either discarded or transferred to a new tube. SDS (1% final concentration) was added to all fractions which were then heated in a water-bath at 94°C for 2 min. The samples were cooled to 23°C and diluted with 1 ml of 1% Triton X-100, 0.2 M NaCl, 12.5 mM sodium phosphate (pH 7.6) and centrifuged at 17,300 *g* for 45 min. Supernatant fractions were transferred to microfuge tubes which contained 40 μg of anti-TNP IgG, incubated for 16 h at 0°C, and then supplemented with 50 μl of IgG Sorb (10% suspension). After 30 min at 0°C the immune precipitates were sedimented and washed four times with 0.7 ml of 0.1% SDS, 0.05% Nonidet P-40, 0.3 M NaCl, 10 mM Tris·Cl (pH 8.6). Final pellets were resuspended in a volume of SDS-gel sample buffer that was proportional to the incorporation of $^{35}SO_4^{2-}$ into cells; this corrected for variations in the rate of protein synthesis among different cultures. Suspended immunoprecipitates were heated in a boiling water bath for 3 min, IgG Sorb was sedimented, and the supernatant samples were transferred to new tubes. Aliquots (30 μl) were analyzed by SDS-gel electrophoresis. Gels were processed for fluorography with Enhance and exposed on prefogged Kodak X-Omat R film.

CONA-SEPHAROSE BINDING: ConA-Sepharose was washed twice with 0.5 M NaCl, 0.5 M α-methylmannoside, four times with 2.5% Triton X-100, 1 mM $MgCl_2$, 0.5 M NaCl, 20 mM triethanolamine (pH 7.5), and resuspended in two volumes of the Triton buffer. A 10-μl aliquot of an SDS solubilized immunoprecipitate was added to a tube containing 200 μl of the Triton buffer with 33 μl or 66 μl of the ConA-Sepharose suspension, and the mixture was incubated at 25°C for 3 h with agitation. The ConA-Sepharose was then sedimented and washed three times with 0.5 ml of 0.05% SDS, 0.5 M NaCl, 20 mM triethanolamine (pH 7.5), and the final pellet was resuspended in 50 μl of SDS-gel sample buffer and heated in a water bath at 94°C for 2 min. After sedimentation of the Sepharose, the supernatant solution was transferred to a new tube and aliquots were analyzed by SDS-gel electrophoresis as described earlier.

RESULTS

Identification of Cell Surface Proteins

Yeast cells are surrounded by a rigid but porous wall consisting of glucan, β-1,3 and β-1,6 polymers of glucose, and mannan, a term given to a collection of secreted glycoproteins which contain large mannose-rich oligosaccharides. The plasma membrane forms the primary permeability layer of the cell. Secreted proteins, such as invertase and acid phosphatase, are trapped between the cell wall and the plasma membrane.

Cell surface proteins can be radiolabeled by catalytic iodination of intact yeast cells. Log-phase cells were labeled with ^{125}I and extracts were prepared by agitation of cells with glass beads. Iodinated proteins were solubilized by heating the extract in the presence of SDS, and analyzed by SDS-gel electrophoresis. Radioactive proteins with mobilities corresponding to 220, 155, 75, 51, 49, 33, 29, and 22 kdal were detected in addition to material which did not migrate beyond the 5% stacking gel (Fig. 1).

Secreted and cell wall proteins are released and spheroplasts are formed when the β-1,3 glucan layer is degraded by a lytic glucanase in the presence of reducing agent and osmotic support (19). Iodinated cells were converted to spheroplasts with a purified glucanase or with lyticase (a lytic enzyme fraction that contains protease and glucanase activities (19). Spheroplasts were sedimented, lysed by osmotic shock, and the resulting lysates were centrifuged to generate membrane and soluble cytoplasmic fractions. The secreted, membrane, and cytoplasmic fractions from each lytic enzyme digest were ex-

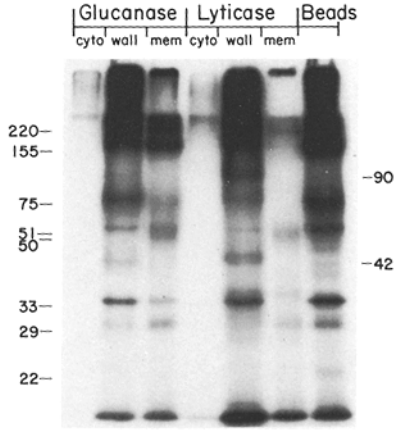

FIGURE 1 Fractionation of iodinated cells. X2180-1A cells (20 A_{600} U) grown in low-sulfate medium at 25°C, were sedimented, resuspended in 7.5 ml of fresh medium, and incubated for 65 min at 37°C. Washed cells were iodinated, divided into three aliquots, and sedimented. Two of the cell pellets were resuspended in 100 μl of spheroplasting medium with either 150 U of purified lytic glucanase or 500 U of lyticase, and incubated at 30°C for 1 h. Secreted and membrane fractions were prepared for gel electrophoresis as in the TNBS tagging procedure described in Materials and Methods. The third cell pellet was mixed with 0.3 g of glass beads (0.5 mm) and 50 μl of 2% SDS, 50 mM sodium phosphate (pH 7.5), 50 mM β-mercaptoethanol. Cells were broken by agitation on a vortex mixer for 1.5 min, followed by heating at 94°C for 2 min. All solubilized fractions were diluted with sample buffer to a final volume of 1 ml, heated at 94°C for 2 min, and aliquots (30 μl) were applied to an SDS gel. An autoradiograph of the gel is shown.

amined by SDS gel electrophoresis. The purified glucanase released the 75 and 51 kdalton species as well as the material that remained in the 5% stacking gel. The membrane fraction contained the 220, 155, 49, and 29 kdal species. A 33 kdal species was found mainly in the secreted fraction, with some in the membrane fraction. This species is found in purified plasma membrane fractions (J. Tschopp and R. Schekman, manuscript in preparation). Lyticase treatment produced an altered pattern of secreted proteins: the 220, and 155 kdal species were diminished and new forms with mobilities of 90 and 42 kdal were observed. Cleavage of cell wall proteins by the lytic protease component of lyticase could account for the altered gel profile. For this reason, all further analysis was conducted with the purified glucanase. As expected, no iodinated proteins were detected in cytoplasmic fractions.

Isolation of Cell Surface Proteins

To assess transport of proteins to the yeast cell surface, we have modified the technique developed by Kaplan, Unkeless, and Cohn (16). Yeast cells were radiolabeled with $^{35}SO_4^{2-}$, sedimented, washed, and treated with TNBS at 0°C. TNBS does not readily penetrate across the plasma membrane at 0°C but will covalently modify cell surface proteins. Radiolabeled, TNBS-tagged cells were resolved into secreted, membrane, and cytoplasmic fractions as described in the previous section. Each sample was treated with SDS, heated, and TNP-tagged proteins were precipitated with anti-TNP IgG and *Staph A* cells. Immunoprecipitates were solubilized, and radioactive proteins were separated by SDS-gel electrophoresis and visualized by fluorography. Fig. 2 (post-tagged) shows that the secreted (wall) and membrane fractions contained distinct species (secreted, $S_1 - S_4$; membrane, $M_1 - M_7$) that corresponded in size to proteins detected by iodination of cells. Proteins S_4 and M_4 had similar SDS gel mobilities; these bands could represent one protein that is largely but not completely released during spheroplast formation. The cytoplasmic fraction had no TNP-tagged proteins, even though before immunoprecipitation 60% of the radioactive protein was in this sample.

To eliminate the possibility that TNP-tagged nonradioactive proteins aggregate with untagged radioactive proteins, we performed a control experiment in which cells were first treated with TNBS, washed, and then metabolically labeled with $^{35}SO_4^{2-}$. These cells contain proteins tagged with TNP and other proteins labeled with ^{35}S but no proteins both tagged and radiolabeled. Aggregation was not a problem because neither the secreted nor the membrane fraction contained immunoprecipitated radioactive proteins (Fig. 2, pretagged). Addition of 50 mM β-mercaptoethanol during solubilization of the membrane fraction was essential for the success of this control. Nonspecific precipitation of radioactive proteins was reduced further by dilution of treated cells with a 10-fold excess of untreated cells before fractionation.

Thermoreversible Export in Secretory Mutants

Wild-type and *sec* mutant cells were radiolabeled at a permissive temperature (25°C) and the pattern of exported proteins was examined by the TNBS-tagging procedure. Fig. 3 shows that the pattern of secreted (wall) and of plasma membrane surface proteins was similar in wild-type and mutant

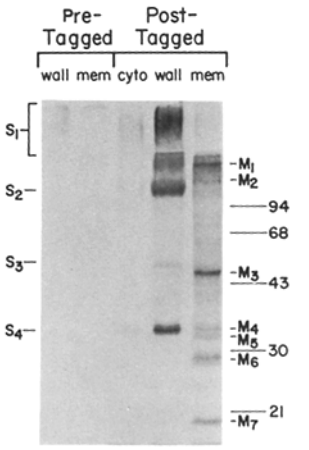

FIGURE 2 TNBS pretagged and posttagged wild-type cells. X2180-1A cells were grown in low-sulfate medium at 25°C, and two aliquots (0.67 A_{600} U cells each) were centrifuged. One cell pellet was resuspended and tagged with TNBS. These cells were then washed, resuspended in 0.25 ml of fresh medium, and labeled with 36 μCi of $^{35}SO_4^{2-}$ for 1 h at 37°C (pretagged cells). The other cell pellet was first labeled with $^{35}SO_4^{2-}$ and then tagged with TNBS (posttagged cells). Cells were fractionated and aliquots of solubilized samples were analyzed by SDS gel electrophoresis; a fluorograph of the gel is shown.

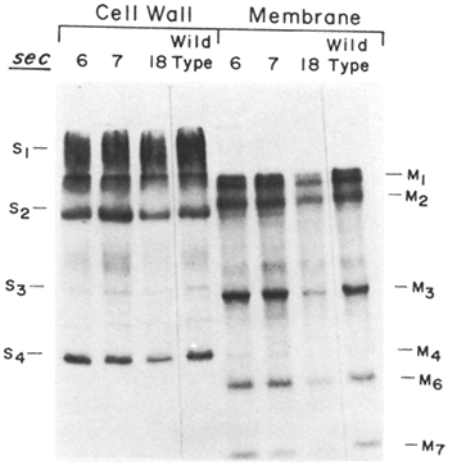

FIGURE 3 Mutant and wild-type cells labeled at 25°C. Mutant (*sec6*, *sec7*, *sec18*) and X2180-1A cells were grown in low-sulfate medium at 25°C, labeled with 100 μCi of $^{35}SO_4^{2-}$ for 1 h at 25°C, tagged with TNBS, and fractionated. Solubilized immunoprecipitate fractions were analyzed by SDS gel electrophoresis; a fluorograph of the gel is shown.

cells at 25°C. At the nonpermissive temperature (37°C), however, export of all major surface proteins was blocked in *sec* mutant cells (Fig. 4). Mutants *sec1* (accumulates secretory vesicles), *sec7* (accumulates Golgi bodies), and *sec18* (accumulates endoplasmic reticulum) showed no detectable export of proteins S_1 to S_4, although a return to 25°C (in the presence of cycloheximide) allowed export (Fig. 4*A*). As was shown for invertase (15), reversible secretion of proteins S_1 to S_4 was blocked by an energy poison. Proteins M_1 to M_7 at the plasma membrane surface were exported at a much reduced level in mutants *sec1* and *sec7* at 37°C but were restored upon return to 25°C (Fig. 4*B*). The M_3 species appeared not to be reduced as completely as the other major proteins in the *sec1* and *sec7* membrane fraction.

The pattern of export at 37°C was examined in representatives from all *sec* complementation groups and, although some were not blocked completely, most strains showed substantial reduction of all major surface proteins. Two exceptions were noted. *sec11*, a mutant which blocks secretion of acid phosphatase more completely than invertase (13), also showed anomalous behavior of the TNP-tagged secreted proteins: export of proteins S_3 and S_4 was blocked, while proteins S_1 and S_2 were secreted normally (data not shown). The membrane fraction from some of the mutants showed a series of TNP-tagged radioactive proteins that were not prominent in wild-type cells labeled at 37°C or in mutant cells labeled at 25°C. The intensity of these radioactive bands varied considerably. Certain mutants, such as *sec1* and *sec7* (Fig. 4*B*), displayed low levels of these proteins at 37°C, while in other mutants, such as *sec18*, these bands were as prominent as the M_1 to M_7 species seen in wild-type cells (not shown). Similarly, a series of new bands appeared in the secreted fraction when *sec18* cells were radiolabeled at 37°C (Fig. 4*A*). The abnormal proteins detected in the mutants may not be at the cell surface, or they may not be apparent unless the export of major proteins is blocked. Evidence suggesting an intracellular location for these proteins is reviewed in the Discussion.

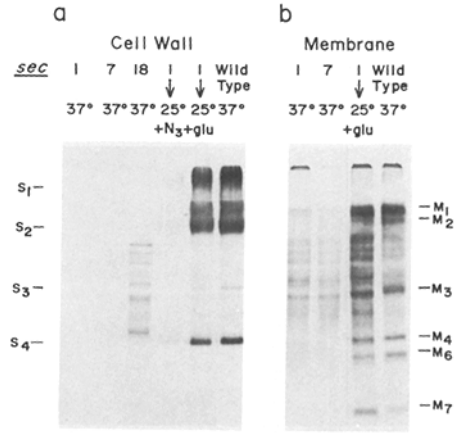

FIGURE 4 Mutant and wild-type cells labeled at 37°C. Cells were grown in low-sulfate minimal medium and labeled with 150 μCi of $^{35}SO_4^{2-}$ for 1 h at 37°C. Two additional cultures of *sec1* cells were labeled and centrifuged; one cell pellet was resuspended in medium containing 0.1 mg/ml of cycloheximide, the other in medium with cycloheximide and 20 mM sodium azide but without glucose. These two cultures were incubated at 25°C for 2 h while the other samples were washed and stored at 0°C. All samples were then tagged with TNBS, fractionated, and solubilized immunoprecipitates (20-μl aliquots) were analyzed by SDS gel electrophoresis. (*a*) Cell wall fraction from: *sec1* 37°C; *sec7* 37°C; *sec18* 37°C; *sec1* 37°C shifted to 25°C with sodium azide, cycloheximide, without glucose; *sec1* 37°C shifted to 25°C with cycloheximide; X2180-1A 37°C. (*b*) Membrane fraction from: *sec1* 37°C; *sec7* 37°C; *sec1* 37°C shifted to 25°C with cycloheximide; X2180-1A 37°C.

Glycosylation and Export of Surface Proteins

The glycoprotein nature of isolated surface proteins was examined by binding to ConA-Sepharose. Wild-type cells were radiolabeled, tagged with TNBS, and TNP-modified proteins isolated with antibody. Immunoprecipitates were solubilized with SDS, diluted with buffer containing Triton X-100, and mixed with ConA-Sepharose. Proteins bound to ConA-Sepharose were sedimented, solubilized again with SDS, and analyzed by SDS-gel electrophoresis. Fig. 5 shows an SDS gel fluorograph in which ConA-Sepharose-bound samples were compared with equal fractions of the original immunoprecipitates. Proteins S_1 to S_4 and M_2 and M_4 were efficiently retained by ConA-Sepharose. Proteins M_1, M_3, and M_6 showed partial binding, while M_5 and M_7 did not bind. Addition of more ConA-Sepharose did not enhance the recovery of any protein. In contrast, among the minor proteins recovered from the membrane fraction of *sec6* cells (accumulates secretory vesicles) labeled at 37°C, none were bound by ConA-Sepharose.

The role of N-linked glycosylation in export of yeast surface proteins was examined with tunicamycin, a drug that blocks synthesis of the core oligosaccharide (22). Treatment of wild-type cells with tunicamycin during the period of radiolabeling resulted in altered electrophoretic profiles for the secreted and membrane fraction immunoprecipitates (Fig. 6). Tunicamycin treatment affected surface proteins in different ways. Protein S_4 appeared to be secreted normally but with a reduced molecular weight; protein M_2 appeared not to be exported; and protein M_6, which did not bind to ConA, appeared to be

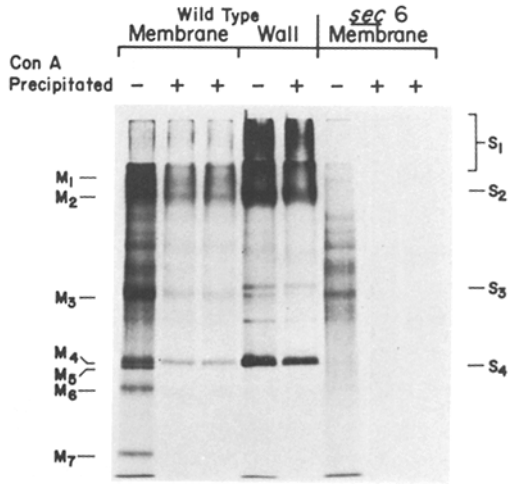

FIGURE 5 Binding of radiolabeled, TNP-tagged proteins to ConA-Sepharose. Solubilized immunoprecipitates, prepared from samples such as in Fig. 4, were diluted and treated with ConA-Sepharose. Bound material was released from the Sepharose and compared in adjacent lanes of an SDS gel with an equivalent volume of the original immunoprecipitate. A fluorograph of the gel shows (starting at the left): (*a*) immunoprecipitate from X2180-1A membrane fraction; (*b*) same material bound to ConA-Sepharose (66 μl); (*c*) same bound to ConA-Sepharose (33 μl); (*d*) immunoprecipitate from X2180-1A cell wall fraction; (*e*) same bound to ConA-Sepharose (66 μl); (*f*) immunoprecipitate from a *sec6* membrane fraction; (*g*) same material bound to ConA-Sepharose (66 μl); (*h*) same bound to ConA-Sepharose (33 μl).

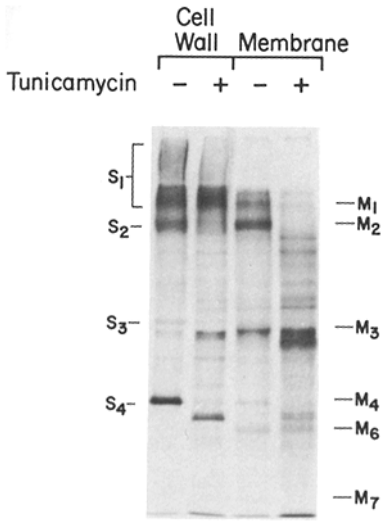

FIGURE 6 Proteins exported in the presence of tunicamycin. X2180-1A cells (0.67 A_{600} U) were suspended in two cultures of minimal medium; one contained tunicamycin (20 μg/ml). After 30 min at 25°C, 190 μCi of $^{35}SO_4^{2-}$ was added to each, and labeling continued for 45 min at the same temperature. Cells were tagged, fractionated, and solubilized material was immunoprecipitated. Aliquots of the solubilized immunoprecipitates were analyzed by SDS gel electrophoresis; a fluorograph of the gel is shown.

exported normally with no change in mobility. It was not always clear whether a protein species was shifted or not exported; however, unlike the effect of the *sec* mutations, protein export was not generally blocked by inhibition of oligosaccharide synthesis.

Transit Time and Turnover of Surface Proteins

The transit time of newly synthesized protein to the cell surface was analyzed in a pulse-chase experiment. Wild-type cells were radiolabeled with $^{35}SO_4^{2-}$ for 2 min and transferred to nonradioactive medium for various chase periods. Cells were tagged with TNBS, fractionated, and analyzed as before. Fig. 7 shows that proteins S_2, M_3, M_5, M_6, and M_7 appeared at the cell surface between 3 and 8 min of chase. By 30 min, proteins S_1, S_3, S_4, and M_1 emerged. Proteins M_2 and M_4 appeared last and continued to increase in intensity between 58 and 88 min of chase.

The asynchronous appearance of surface proteins implied a differential delay in transport along the secretory pathway. *sec* Mutants blocked early (*sec18*, ER-blocked) and late (*sec6*, secretory vesicle-blocked) in the secretory pathway were used to evaluate the site of delay. Cells were labeled with $^{35}SO_4^{2-}$ for 4 min at 37°C and then chased at the same temperature for 45 min in nonradioactive medium. Aliquots were withdrawn at various times after return to 25°C, and secreted proteins were analyzed by TNBS tagging. If asynchrony developed before the secretion block, the chase at 37°C could allow proteins to be exported with similar kinetics during recovery at 25°C. Fig. 8 shows that, in *sec6*, proteins S_2 and S_4 were released coordinately at 25°C. Proteins M_1, M_2, and M_3, which appeared asynchronously in the experiment in Fig. 7, were exported coordinately in the *sec6* experiment (not shown). The same experiment with *sec18* showed a lag between proteins S_2 and S_4 just as was seen in the pulse-chase labeling of wild-type cells (data not shown). These results suggested that protein S_4 was delayed either in the ER (after the *sec18* block) or in the Golgi body. Unfortunately, *sec7*, the Golgi-blocked mutant, was not thermoreversible under the conditions of this experiment (15).

Turnover of cell surface proteins was examined with ^{125}I-labeled cells that were TNBS-treated before or after one generation of growth. TNP-tagged and iodinated cells were fractionated and aliquots of each sample were analyzed before and after precipitation with TNP-antibody. At time zero, 46% of the cell wall protein ^{125}I and 73% of the plasma membrane surface ^{125}I were immunoprecipitated. SDS gel analysis (Fig. 9) showed virtually identical patterns of total and TNP-tagged iodinated proteins. The principal loss of ^{125}I label in the cell wall fraction was due to incomplete precipitation of protein S_1. After growth for 2 h (one doubling time), the yield of surface

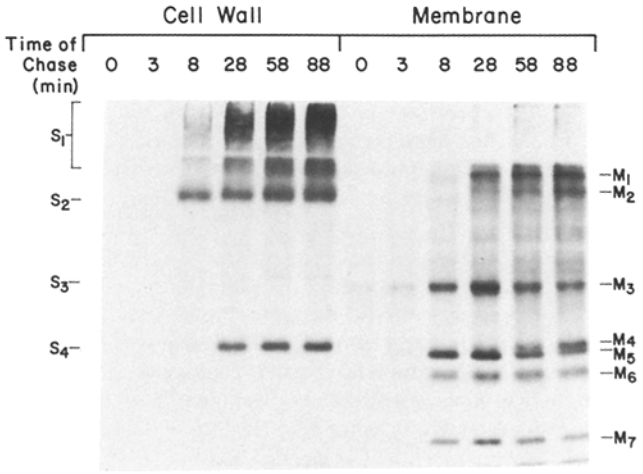

FIGURE 7 Transit time of cell surface proteins. X2180-1A cells (4 A_{600} U in 1 ml), grown in low-sulfate minimal medium at 25°C, were incubated at 37°C for 5 min and then labeled with 1.5 mCi of $^{35}SO_4^{2}$ for 2 min. Ammonium sulfate (0.5 mM final concentration) was added and the cells were sedimented, resuspended in 1.5 ml of fresh medium, and incubation was continued at 37°C. At the indicated times, 0.25-ml samples were removed and chilled to 0°C. At the end of the experiment, all samples were processed for TNBS tagging. The chase was effective because the amount of total radioactive protein did not vary by >6% from the zero-time sample. Aliquots of solubilized immunoprecipitates from the cell wall and membrane fractions were analyzed by SDS gel electrophoresis; a fluorogram of the gel is shown.

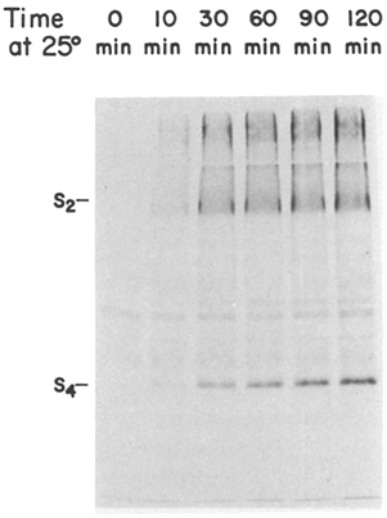

FIGURE 8 Synchronous export of secretory proteins accumulated in *sec6*. *sec6* Cells (4 A_{600} U in 1 ml), grown in low-sulfate minimal medium at 25°C, were incubated at 37°C for 2 min and then labeled with 0.4 mCi of $^{35}SO_4^{2-}$ for 4 min. Cells were sedimented, resuspended in 15 ml of minimal medium containing 0.5 mM ammonium sulfate, and incubation was continued for 45 min at 37°C. Cycloheximide (0.1 mg/ml) was added and the culture was cooled to 25°C. At the indicated times, 0.25-ml aliquots were removed and chilled to 0°C. At the end of the experiment, all samples were processed for TNBS tagging. Aliquots of solubilized immunoprecipitates from the cell wall fraction were analyzed by SDS gel electrophoresis; a fluorogram of the gel is shown.

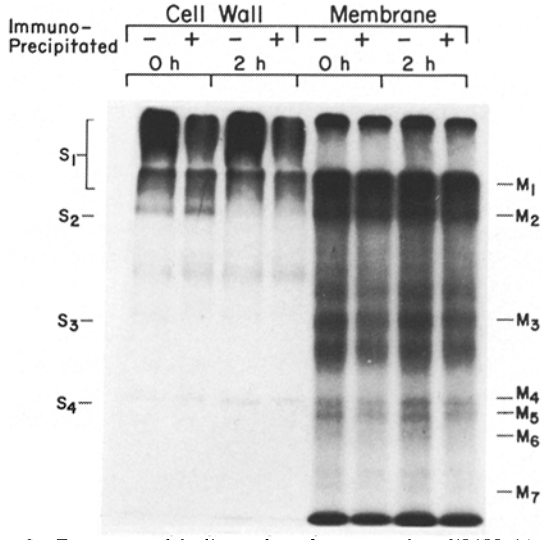

FIGURE 9 Turnover of iodinated surface proteins. X2180-1A cells (4.5 A_{600} U), grown in low-sulfate minimal medium, were iodinated and resuspended in 1.1 ml of fresh medium. Two aliquots (0.25 ml) were removed, washed, and stored at 0°C. The remaining culture was incubated for 2 h at 37°C; growth resumed after a 15 min lag. Two aliquots (0.25 ml and 0.125 ml) of the 2-h culture were taken and all samples were washed, tagged, fractionated, and solubilized in SDS. A 0 time, and the larger 2-h sample, were diluted to 1 ml with SDS gel sample buffer. The tagged proteins were immunoprecipitated from the remaining samples, then solubilized in either 1 ml (0 time sample) or 0.5 ml (2-h sample). Aliquots (35 μl) of all samples were analyzed by SDS gel electrophoresis; an autoradiograph of the gel is shown.

protein was unchanged: 44% recovery of cell wall label, and 76% recovery of plasma membrane label. Only protein S_2 disappeared during the growth period.

DISCUSSION

We have demonstrated common genetic requirements for export of the major secreted (cell wall) and externally exposed plasma membrane proteins in yeast. Although there appears to be one major transport pathway, proteins move to the cell surface at different rates and are affected unequally by inhibition of glycosylation. These differences may reflect variations in the interaction of exported proteins with the processing, sorting, and transport machinery, rather than alternate pathways of localization.

The components of the yeast cell surface have been defined by two techniques. The first, radioiodination of intact cells, identifies surface proteins with exposed tyrosine residues. The other, TNBS treatment and TNP-antibody precipitation, allows identification and isolation of radiolabeled surface proteins that have reactive amino groups. Both probes label secreted and plasma membrane surface proteins but not cytoplasmic proteins. The cell wall fraction contains proteins with apparent molecular weights of >250 kdal (S_1; could be several protein species), 140 (S_2), 51 (S_3), and 33 kdal (S_4). Iodination reveals a 75 kdal protein that is detected only marginally by the TNBS procedure with cells that are radiolabeled at 25°C (compare Figs. 1 and 3). The membrane fraction contains proteins with apparent molecular weights of 220 (M_1), 155 (M_2), 49 (M_3), 33 (M_4), and 29 kdal (M_6). In addition, a 31 kdal protein (M_5) can often be resolved from protein M_4 (Fig. 2), although synthesis of both M_4 and M_5 is reduced at 25°C (compare Figs. 2 and 3). A protein with an apparent molecular weight of 19 kdal (M_7) is detected by the TNBS procedure but not by iodination. A good correlation between the two techniques is most apparent from a comparison of ^{125}I-labeled surface proteins from TNBS-tagged cells before and after TNP-antibody precipitation (Fig. 9). When the yield of ^{125}I in the TNP-antibody immunoprecipitate is taken into account, the cell wall protein accounts for 0.9%, and the plasma membrane surface protein accounts for 0.5%, of the total $^{35}SO_4^{2-}$ incorporated into cells during a 1-h incubation.

The TNBS procedure has allowed an evaluation of protein export in mutants that are temperature-sensitive for secretion and growth. Earlier work on the *sec* mutants suggested a pleiotropic defect in export of cell wall glycoproteins and a plasma membrane permease activity (13). The results presented here demonstrate that the conditional block is a general phenomenon. Furthermore, with the exceptions noted below, representative mutant alleles of 22/23 class A *sec* genes show the same pattern of blocked transport. In some of the mutants, export of the major surface proteins is reduced but not eliminated. This is best explained by mutations that are not completely restrictive at 37°C. On the other hand, *sec11* blocks export of two secreted proteins, with no effect on two others. This mutant also shows unequal effects on secretion of acid phosphatase and invertase and, unlike the other *sec* mutants, *sec11* does not accumulate organelles (13). The *SEC11* gene may only be required for export of a subset of cell surface proteins.

The membrane fraction from *sec* mutant cells radiolabeled at 37°C contains TNP-tagged proteins that are less apparent in a comparable wild-type cell preparation, or in *sec* mutant cells labeled at 25°C (Figs. 3 and 4). Either these proteins are not really exposed at the cell surface and are recovered in the membrane fraction because of some artifact, or they are surface proteins that become apparent only when the export of major surface proteins is blocked. Several lines of indirect evidence support the first explanation. First, unlike many of the wild-type membrane surface proteins, none of the labeled proteins in a *sec6* membrane fraction binds to concanavalin A (Fig. 5). Second, the labeling of these anomalous proteins varies irregularly among *sec* mutant strains; there is no pattern of appearance with respect to the stage in the secretory pathway that is blocked. Third, the *sec* mutants that show pronounced labeling of these species also reveal a series of new polypeptides in the cell wall fraction (*sec18*, Fig. 4*A*). We suspect that the anomalous proteins are of cytoplasmic origin. A small and variable percentage of *sec* mutant cells may become permeable during incubation at 37°C so that subsequent TNBS treatment allows tagging of intracellular proteins. Although the results with mutants *sec1* and *sec7* (Fig. 4) clearly indicate that transport of the major surface proteins is blocked at 37°C, the possibility that a minor class of membrane proteins is exported by a different mechanism deserves further analysis with more specific probes.

We have previously shown that the organelles and glycoproteins accumulated in single and double *sec* mutant strains define a unique, linear secretory pathway (14, 15). Although the TNBS-tagging experiments support this conclusion, the results are also consistent with parallel pathways in which subsets of exported proteins travel in different compartments. This possibility will be tested by analysis of secretory organelles purified from *sec* mutant cells.

Alternate, or parallel, transport pathways have been invoked to explain the widely varying rates at which newly synthesized proteins are exported. Strous and Lodish (9) reported transit

times of 23 min for albumin and VSV G protein and 40 min for transferrin in rat hepatoma cells. Monensin causes transport of transferrin to arrest before, and VSV G protein after, conversion of *N*-glycosidically linked oligosaccharides from the early high-mannose to the mature complex form. In spite of these differences, the two proteins accumulate in the same Golgi body vesicles (H. Lodish, personal communication). More dramatic, however, is the recent demonstration of two pathways for the secretion of ACTH (11). A pituitary cell line secretes mature ACTH by regulated exocytosis of granules; the transit time of mature ACTH in unstimulated cells is 3–4 h. The same cells display constitutive export of a viral membrane glycoprotein, and the precursor of ACTH, with a transit time of 40 min. Furthermore, the granules responsible for secretion of mature ACTH have been purified and contain neither ACTH precursor nor viral glycoprotein. Gumbiner and Kelly propose a branch point at the Golgi body to account for regulated and constitutive limbs of the secretory pathway.

There is no precedent for regulated secretion in yeast. Thin sections of budding yeast cells show very low levels of secretory organelles (12). Transport rates in yeast are generally greater than in mammalian cells: yeast invertase transits in 5 min or less (15), and a protease secreted by *Saccharomycopsis lipolytica* requires only 3 min for transport (D. Ogrydziak, personal communication). The TNBS-tagging procedure has revealed major yeast surface proteins that are secreted rapidly, like invertase, and others that emerge more slowly (Fig. 7). The asynchrony appears to develop at a late step in the ER, or in the Golgi body. When *sec* mutant cells are pulse-labeled and chased at 37°C, synchronous secretion occurs upon return to 25°C if the mutant is blocked after, but not before, the Golgi body stage (Fig. 7). A similar conclusion was drawn by Strous and Lodish (9) who found that pulse-labeled glycoprotein precursors show transit time-dependent variation in the rate of oligosaccharide processing, reflecting asynchronous passage through the Golgi body.

Although many of the yeast surface proteins are glycosylated, as judged by their binding to ConA (Fig. 5), glycosylation appears not to be a general requirement for export (Fig. 6). A 30 kdal polypeptide, perhaps related to protein S_4, is synthesized and secreted when cells are treated with tunicamycin. Protein M_2, on the other hand, appears not to be exported without glycosylation. This heterogeneity in the effect of tunicamycin-treatment on protein transport has been noted in other systems (9, 23). Glycoproteins may vary in the degree to which oligosaccharides participate in protein folding, and this may be reflected in the rate of transport through the secretory pathway.

Turnover of yeast cell surface proteins is slow with respect to the generation time. All but one surface protein remains in place during one cell-doubling time (Fig. 9). Although endocytosis has not been examined in yeast cells, the possibility remains that surface proteins are subject to internalization and recycling.

We wish to thank Dr. Ann Good for advice and for a generous supply of anti-TNP serum, Dr. J. Unkeless for emphasizing the importance of the pretagging control, and Susan Ferro-Novick for advice on the ConA-Sepharose binding experiment.

This work was supported by grants from the National Institutes of Health (GM-26755 and ES01896) and the National Science Foundation (PCM 80-19415).

Received for publication 9 August 1982, and in revised form 20 October 1982.

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