New Mutants of *Saccharomyces cerevisiae* Affected in the Transport of Proteins From the Endoplasmic Reticulum to the Golgi Complex

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ABSTRACT

We have isolated new temperature-sensitive mutations in five complementation groups, *sec31–sec35*, that are defective in the transport of proteins from the endoplasmic reticulum (ER) to the Golgi complex. The *sec31–sec35* mutants and additional alleles of previously identified *sec* and vacuolar protein sorting (*vps*) genes were isolated in a screen based on the detection of α-factor precursor in yeast colonies replicated to and lysed on nitrocellulose filters. Secretory protein precursors accumulated in *sec31–sec35* mutants at the nonpermissive temperature were core-glycosylated but lacked outer chain carbohydrate, indicating that transport was blocked after translocation into the ER but before arrival in the Golgi complex. Electron microscopy revealed that the newly identified *sec* mutants accumulated vesicles and membrane structures reminiscent of secretory pathway organelles. Complementation analysis revealed that *sec32–1* is an allele of *BOS1*, a gene implicated in vesicle targeting to the Golgi complex, and *sec33–1* is an allele of *RET1*, a gene that encodes the α subunit of coatomer.

The secretory pathway of eucaryotic cells consists of a series of distinct membrane-bound organelles through which exported proteins pass en route to the cell surface (Palade 1975). Genetic analysis of the secretory pathway in the yeast *Saccharomyces cerevisiae* has allowed the identification of essential gene products that function in this pathway, many of which are conserved (reviewed in Pryer et al. 1992; Wuestehube and Schekman 1993). A large number of temperature-sensitive mutations that disrupt secretory protein transport upon shift to the nonpermissive temperature have been isolated (Novick et al. 1980). These secretory (*sec*) mutations were shown to block secretion at three phenotypically distinguishable stages of the pathway using both morphological and biochemical criteria. The first class of *sec* mutants produced an extended network of endoplasmic reticulum (ER) membrane and accumulated core-glycosylated protein precursors. The second class formed stacked membrane structures reminiscent of the mammalian Golgi complex and accumulated heterogeneously glycosylated protein precursors. The third *sec* mutant class accumulated 80–100 nm secretory vesicles and nearly fully glycosylated precursors. Epistatic relationships were established by examining the morphology of double mutants (Novick et al. 1981). The mutants that displayed the ER-accumulating phenotype were epistatic to those that displayed the stacked membrane and vesicle-accumulating phenotypes, and mutants that displayed the stacked membrane phenotype were epistatic to those that displayed the vesicle-accumulating phenotype. Thus, the temporal and spatial organization of the secretory pathway has been defined by phenotypic characterization of the *sec* mutants.

The first stage of the secretory pathway, that of transport from the ER to the Golgi complex, has been the focus of intense study. Temperature-sensitive mutations have been isolated in 13 genes involved in this step, including *SEC12/SED2*, *SEC13*, *SEC16*, *SEC17*, *SEC18*, *SEC20*, *SEC21*, *SEC22/SLY2*, *SEC23*, *BET1/SLY12*, *BET2*, *YPT1*, *USO1* (Novick et al. 1980; Newman and Ferro-Novick 1987; Schmitt et al. 1988; Segev et al. 1988; Bacon et al. 1989; Nakajima et al. 1991). Other genes important for ER to Golgi transport have been identified by high copy suppression screens, including *BOS1*, *SARI*, *SLY1*, and *SED5* (Nakano and Muramatsu 1989; Newman et al. 1990; Ossig et al. 1991; Hardwick and Pelham 1992). Biochemical analysis of the products encoded by these genes has been facilitated by the development of cell free assays that reconstitute ER to Golgi transport (reviewed in Pryer et al. 1992). Several gene products have been shown to function in vitro as multisubunit complexes. In addition to known *SEC* gene products, these complexes were composed of other proteins required for ER to Golgi transport that had not been previously identified by genetic means (Hicke et al. 1992; Pryer et al. 1993; Salama et al. 1993).

The *SEC* genes required for protein traffic between the ER and Golgi complex have been subdivided into interacting groups using epistasis tests and synthetic-lethal genetic interactions (Kaiser and Schekman 1990). One group of *SEC* genes functions in the formation of ER-derived transport vesicles and includes...
MATERIALS AND METHODS

Strains and culture methods: Genotypes of the yeast strains used in this study are listed in Table 1. Yeast crosses, complementation tests and meiotic segregation analyses were performed using standard genetic techniques (Rose et al. 1989).

For mutant isolation, wild-type strain RSY255 was mutagenized to 50% viability by spreading ~7500 cells from each of 10 independent stationary cultures onto YPDagar plates (150 mm diameter) and irradiating with UV light. After UV light exposure mutagenized cells were shielded from light for ~24 hr. Each mutant was crossed two or three times against the congenic wild-type strain RSY259 to minimize genetic variation between strains. Strains of a mating type for complementation studies were obtained as segregants of the mating RSY259 × sec mutant strain. Double mutants were obtained as segregants of the mating sec mutant × sec mutant, and their genotypes were checked by complementation analysis against each parental allele to ensure the retention of both alleles.

Media for growth of yeast were prepared as described (Sherman 1991). YP medium contained 1% Bacto-yeast extract and 2% Bacto-peptone (Difco Laboratories, Detroit, MI); YP medium was the same with 2% glucose. YPd-agar was supplemented with 2% Bacto-agar (Difco). Minimal medium contained 0.67% Bacto-yeast nitrogen base without amino acids or ammonium sulfate and 2% glucose. The optical density at 600 nm (OD600) of dilute cell suspensions was measured in 1-cm quartz cuvettes in a Zeiss PMQII spectrophotometer (Carl Zeiss, Inc., Thornwood, NY). One OD600 unit of cells corresponds to ~1 x 10^6 cells or 0.15 mg dry weight.

Colony immunoblotting for a-factor precursor: Elevated levels of a-factor precursor were detected by immunoblotting of yeast colonies transferred to nitrocellulose filters. Colonies were grown on YPD-agar plates at 24°C to ~1 mm in diameter then replica plated to nitrocellulose filters (0.45 mm, Schleicher & Schuell, Keene, NH) that were placed onto fresh YPD-agar plates and shifted to 38°C for 3 hr. Cells were lysed by placing the nitrocellulose filters onto Whatman paper soaked with 0.1% SDS, 0.2 M NaOH, 35 mM dithiothreitol (DTT) for 30 min. After removal of lysed cells by rapidly flowing distilled water, nitrocellulose filters were treated with 0.1 M acetic acid for 30 min to inactivate endogenous alkaline phosphatase, rinsed with 50 mM Tris pH 7.4, 150 mM NaCl, and then were incubated in milk buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% NP-40, 2% nonfat dry milk) for ~30 min to block nonspecific protein binding sites. Nitrocellulose filters then were incubated with a 1:1000 dilution of affinity-purified anti-a-factor antibody in milk buffer for 2 hr at 24°C, washed three times with milk buffer, and subsequently incubated in 1:5000 alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad, Richmond, CA) for 1 hr at 24°C. Filters were washed three times with milk buffer, once with 50 mM Tris pH 7.4, 150 mM NaCl, and were developed by incubation in 150 µg/ml 5-bromo-4-chloro-3-indolyl phosphate, 300 µg/ml Nitro blue tetrazolium, 0.1 M NaHCO3, 1 mM MgCl2, pH 9.8, for 10–30 min at 24°C. Colonies exhibiting elevated levels of a-factor precursor appeared as purple deposits on the filters. Antibody to a-factor precursor (Rothe1att and Meyer 1986) was affinity-purified as described (Wuestehube and Rothman 1983).

Carboxypeptidase Y (CPY) secretion: Secreted CPY was detected using an immunoblotting technique in which patches of yeast cells grown at 24°C were overlayed with a prewetted nitrocellulose filter (Rothman et al. 1986; Roberts et al. 1991). For this analysis, antibody to CPY was first adsorbed against a column containing lysate proteins of cells lacking CPY. The cell lysate was prepared from stationary phase cultures of PRCl deletion mutant cells and coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ). As a control for cell lysis during blotting, replicate filters were probed with antibody against the cytosolic protein, phosphoglycerate kinase, kindly provided by J. Thorner (this department). Filters were probed with goat-anti-rabbit conjugated to alkaline phosphatase and color development reagents from Bio-Rad as described above.

Invirtase assays: Yeast cells were grown at 24°C in YPD medium to an OD600 of 0.5–1.0 and then shifted to YP medium containing 0.1% glucose at either 24°C or 38°C to induce secreted invertase (Perlman and Halvorson 1981; Carlsson and Botstein 1982). Invertase activity in periplasm and spheroplast fractions was measured to assess secreted and internal invertase, respectively (Goldstein and Lampen 1975). Cells (1 OD600 U) were sedimented in a clinical centrifuge, washed once with distilled H2O, resuspended in YP medium containing 0.1% glucose, and then incubated for 3 hr at either 24°C or 38°C. An equal volume of ice-cold 20 mM NaNO3 was
Strains for crosses were sedimented in a clinical centrifuge, resuspended in 200 μl potassium phosphate pH 7.5, 10 mM NaN₃, 0.1% Triton X-100 and lysed by vigorous vortexing in the presence of glass beads (425–600 mm, Sigma, St. Louis, MO). The spheroplasts were resuspended in 200 μl 50 mM potassium phosphate pH 7.5, 10 mM NaN₃, 0.1% Triton X-100 and resuspended in 200 μl potassium phosphate pH 7.5, 1.4 M sorbitol, 10 mM P-mercaptoethanol containing 100 U reverse transcriptase inhibitor (RTI) and incubated at 37°C for 10 min. N-ethylmaleimide, 200 μM, was added to the second stage of the reaction to prevent interference by residual P-mercaptoethanol. One unit of invertase activity was defined as the amount of enzyme that hydrolyzes sucrose to produce 1 μmol glucose per minute at pH 5.1 that hydrolyzes sucrose to produce 1 μmol glucose per minute at pH 5.1.

### TABLE 1

Yeast strains

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Antisera to a-factor precursor (ROTHBIATT and MEYER 1986), factor precursor, CPY or dipeptidyl aminopeptidase B (DPAP) with the antie1,GMannose serum as described (FRANZUSOFF B). The amount of antiserum per ODs00 cell equivalent was added to a Washed Protein A-Sepharose pellet with bound antigen IP buffer, samples were clarified by centrifugation for 10 min and were resuspended in 50 μl/OD600 cell equivalent with sample buffer. Immunoprecipitation: Yeast cultures were grown to a density of 0.1–0.4 OD600 U/ml at 24° in minimal medium supplemented with 0.2% casamino acids (Difco), appropriate nutrients and 100 μM (NH4)2SO4. An aliquot of cells was sedimented in a clinical centrifuge, washed once and resuspended to 5 OD600 U/ml in minimal medium containing appropriate nutrients, 100 μM (NH4)2SO4, 0.2 mg/ml bovine serum albumin (BSA), 0.1 mg/ml α2-macroglobulin, and incubated at 38° for 1 hr. Radiolabeling was performed in the same medium without (NH4)2SO4 using Express 35S protein labeling mix (L. E. Du Pont, Wilmington, DE) at a level of 30–60 μCi/OD600 cells for 10 min at 38° and chased with 5 mM cysteine, 5 mM methionine, 0.2% yeast extract, 100 μM (NH4)2SO4 at 38°. Reactions were terminated by addition of an equal volume of ice-cold 50 mM Tris-HCl pH 7.5, 40 mM NaCl, 40 mM NaF, 2 M sorbitol, and samples were chilled on ice for 10 min. Radioactive cells were converted to spheroplasts by treatment with 10 mM DTT for 10 min at 30° followed by 30 μl/OD600 cells of recombinant β-1,3 glucanase (SHEN et al. 1991) for 30 min at 30°. Samples were centrifuged and the pellet/lysate fractions by sedimentation in a clinical centrifuge. Spheroplasts were resuspended to 50 μl/OD600 cell equivalent with sample buffer (1% SDS, 10% sucrose, 1 mM EDTA, 30 mM DTT, 40 μg/ml Bromophenol Blue, 10 mM Tris-HCl pH 8.0) and lysed by vigorous vortexing with glass beads. Secreted proteins in the periplasm/media fractions were precipitated by incubation with 10% trichloroacetic acid (TCA) for ≥20 min on ice, washed twice in ice-cold acetone with agitation in a bath sonicator in the presence of glass beads, and resuspended to 50 μl/OD600 cell equivalent with sample buffer. Samples were heated at 70° for 10 min.

Aliquots of spheroplast and periplasm/media samples were diluted in 1 ml IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS) containing 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 1 mg/ml a2-macroglobulin, and incubated at 38° for 1 hr. Radiolabeling was estimated by comparing the total length of intracellular membranes seen in cross sectional view (segments connected to nucleus, in cytoplasm and at cell periphery) to the cell circumference. Since much of the intracellular membrane underlies the plasma membrane at the cell periphery, a fair approximation of membrane lengths relative to cell circumference is obtained. Cell sections were scored using a five-level scale of membrane length:cell circumference ratio as follows: level 1 = <0.50, level 2 = 0.50–0.75, level 3 = 0.75–1.00, level 4 = 1.00–1.25, level 5 = >1.25. A total of 60 random cell sections were scored that consisted of the 30 cell sections that were assayed for vesicle accumulation and 30 additional cell sections from random fields photographed at a lower magnification (4000–9000×). Contact prints of the first set of 30 cell sections and 8 x 10 prints of the second set of 30 cell sections were found to be suitable for this purpose. Although this method provided qualitative, rather than strict quantitative, data, it permitted a more objective comparison of the amount of membrane in wild-type and sec mutant strains.

Suppression and tetrad analysis: Suppression conferred by genes involved in ER to Golgi transport was examined. The sec31–35 mutants were transformed with either single-copies or 2μ-plasmids encoding Sed1p, Sed5p, Syl1p and Bos1p (and control plasmids). In addition, sec33-1 was transformed with plasmids harboring the RET1, SEC21, SEC26 and SEC27 genes. Plasmids were introduced into sec strains by the lithium acetate method (Ito et al. 1985), and transformants were grown on selective minimal plates at 24°. Single colonies were re streaked onto selective minimal plates to maintain selection for the plasmid, and growth at 24°, 30°, 34° and 37° was scored after 3–4 days.

The allelic nature of some genes was tested using tetrad analysis. To determine whether SEC32 was allelic to BOS1, an isolate of sec32-1 (strain JBY1, genotype: MATa, sec32-1, leu2-3,-112) was crossed to a strain in which the BOS1 chromosomal locus was marked by integration of the URA3 plasmid marker (strain JBY2, genotype: MATa, ura3-52, leu2-3,-112, his3-1101, BOS1::URA3). This marked strain was constructed by backcrossing an isolate of betl-1 (strain SNV81, genotype: Matα, bet1-1, ura3-52, his3-1101, BOS1::URA3) to a wild-type strain (strain RSY257, genotype: Matα, ura3-52, leu2-3,-112) to eliminate the bet1-1 mutation and obtain the desired auxotrophic markers. To test whether SEC33 and RET1 were allelic, sec33-1 (strain RSY956, genotype: Matα,
RESULTS

Detection of α-factor precursor by colony immunoblotting: The glycosylation and processing events that occur as the yeast pheromone precursor prepro-α-factor traverses the secretory pathway in yeast have been well documented (Julius et al. 1984; reviewed in Brake et al. 1990). Initially synthesized as a long precursor containing four copies of the mature peptide within it, α-factor precursor is glycosylated in the ER and Golgi complex and cleaved into four peptides by the Kex2 protease in a late Golgi compartment (Franzusoff et al. 1991; Redding et al. 1991). Previous studies have shown that α-factor precursor, but not mature α-factor peptide, absorbs to nitrocellulose (Wilsbach and Payne 1993). We reasoned that the detection of α-factor precursor on nitrocellulose would serve as a powerful primary screen for the isolation of mutants defective in the early stages of the secretory pathway in yeast, which would accumulate the uncleaved α-factor precursor. We therefore developed conditions to identify yeast mutants exhibiting elevated levels of α-factor precursor. In this procedure, yeast colonies were replicated to and lysed on nitrocellulose filters under conditions that allow absorption of solubilized proteins onto nitrocellulose (Lyons and Nelson 1984). The α-factor precursor then was visualized with affinity-purified antibodies using standard immunoblotting techniques. In this screen, we would expect to isolate mutations that block the secretory pathway before the Kex2p-containing late Golgi compartment, including mutations affecting translocation of proteins into the ER, transport from the ER to the Golgi complex, and transport within the Golgi complex. We also would expect to isolate mutations in genes affecting the sorting of proteins through the secretory pathway. For example, α-factor is secreted in a precursor form by some of the vacuolar-protein sorting (vps) mutants (Robinson et al. 1991; Wilsbach and Payne 1993). Since both accumulated as well as secreted α-factor precursor would be detected by the colony immunoblotting screen, mutations in vps genes also would be isolated.

As expected, we found that immunoblotting of yeast colonies for elevated levels of α-factor precursor was most sensitive in detecting mutations that affect the early stages of the secretory pathway (Figure 1). As compared to wild-type (SEC) strains, a strong signal was observed for mutants blocked in translocation into the ER (sec63) and in transport from the ER to the Golgi complex (sec18, sec23). A faint but reproducible signal, most readily seen in individual colonies, was exhibited by mutants defective in intra-Golgi transport (sec7). No signal was observed for mutants blocked late in the secretory pathway (sec1). In addition to α-factor precursor accumulated intracellularly in sec mutants, α-factor precursor that was secreted by yeast colonies also was detected by colony immunoblot. Several vps mutants as well as the clathrin light chain mutant reproducibly gave a signal in this assay (data not shown). These re-
sults indicated that immunoblotting yeast colonies for elevated levels of α-factor precursor detected mutations affecting both transport and sorting of proteins through the secretory pathway.

**Mutant isolation:** Several steps were employed to isolate new temperature-sensitive mutants blocked in the secretory pathway (Table 2). Mutagenized cells were grown to colonies and then tested for elevated levels of α-factor precursor by the immunoblotting method described above. Colonies showing elevated levels of α-factor precursor were purified by streaking onto fresh YPD-agar plates, retested by immunoblotting for α-factor precursor to ensure heritability of this phenotype, and subsequently tested for temperature-sensitive growth. Genetic linkage between the elevated α-factor precursor and temperature-sensitive growth phenotypes then was determined by crossing all haploid mutants to a congenic wild-type strain and performing tetrad analysis. For 104 of the outcrossed mutant strains, a 2:2 segregation for temperature sensitivity to temperature insensitivity was observed in 10–20 tetrads, and in all MATα segregants examined, the α-factor precursor phenotype cosegregated with temperature sensitivity. These results indicated that the elevated levels of α-factor precursor and temperature-sensitive growth phenotypes were the result of a single nuclear lesion in each mutant. Dominance tests were performed by crossing mutants derived from RSY255 to a wild-type strain (RSY259). The growth properties of the resultant heterozygous diploids at 38° indicated that all but one of the mutations was recessive.

The mislocalization of soluble vacuolar proteins to the cell surface is a hall mark phenotype of vps mutants (reviewed in Klionsky et al. 1990). To identify mutations that confer a vps phenotype, we screened directly for the secretion of the soluble vacuolar protease, CPY (Figure 2). A wild-type strain (SEC), two secretory mutants (sec18 and ypt11s) and a vps mutant (vps18) were included as controls. The vps18 mutant gave a very strong signal in this assay. By contrast, the secretory mutants gave a low or nearly undetectable signal, similar to that observed for wild-type cells. In all, we found that 31 of the mutants we isolated secreted CPY at levels comparable to that secreted by previously isolated vps mutants (Table 2). Complementation analysis of the temperature-sensitive growth phenotype demonstrated that ≥20 of these mutants fall into six previously identified VPS complementation groups including VPS11, VPS15, VPS16, VPS18, VPS33, and VPS34. The remaining 73 mutants that did not secrete significant amounts of CPY were thus phenotypically distinct from the vps mutants and were studied further.

Mutations in known secretory genes that were isolated in our screen were identified by complementation analysis. Mutants were crossed with each of 34 previously isolated secretory mutants (listed in Table 1) and the resulting diploids were examined for growth at 24° and 38°. To confirm the allelic nature of mutations that failed to complement, linkage analysis was performed by sporulating the diploids and examining the growth properties of the meiotic segregants. As expected, our screen detected mutations in previously identified genes that function at different early stages of the secretory pathway including ER translocation and processing (KAR2, SEC53, SEC59), ER to Golgi transport (SEC13, SEC16, SEC23, USO1, YPT1), and intra-Golgi transport (SEC7). It is likely that the alleles isolated in this screen affect transport at the same stage of the secretory pathway as previously isolated alleles because many of the mutants accumulate the same incompletely processed precursors of secretory proteins and exhibit similar ultrastructural features (data not shown). Unexpectedly, mutations in genes affecting late stages in the secretory pathway (SEC2, SEC3, SEC5) also were isolated in this screen. It is possible that these

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<td>Complement 34 previously isolated secretory mutants</td>
<td>57</td>
<td>0.15</td>
</tr>
<tr>
<td>Accumulation of CPY core-glycosylated p1 precursor</td>
<td>6</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**TABLE 2**

Unprocessed α-factor colony immunoblot screen for temperature-sensitive secretion mutants

![Figure 2](image-url)
mutations represent new alleles that are blocked in secretion before the Kex2p-containing compartment. However, this appears unlikely since these mutants accumulated an abundance of 80–100 nm vesicles, a phenotype characteristic of post-Golgi blocked secretory mutations (Novick et al. 1981). Alternatively, the mutant alleles of the late-acting secretory genes isolated in this screen may impede the pathway in such a way that incompletely processed α-factor precursor accumulates in these strains.

**Mutations affecting ER to Golgi transport:** The stage in the secretory pathway at which sec mutant cells are arrested was monitored by analyzing the extent of modification and processing of CPY. CPY is synthesized as a 59-kD polypeptide that acquires 10 kD of N-linked carbohydrate (Haslik and Tanner 1978) as it transits through the secretory pathway to the vacuole (Stevens et al. 1982). A 59-kD unglycosylated form of CPY that is cytosolically exposed accumulates in mutants that block translocation into the ER lumen (Deshai and Schekman 1987). An incompletely glycosylated 67-kD form (p1 CPY) accumulates in mutants blocked in transport from the ER, whereas a fully glycosylated 69-kD form (p2 CPY) also accumulates in mutants defective in transport within the Golgi complex (Stevens et al. 1982).

We focused on those mutations that affected the transport of proteins from the ER to the Golgi complex by screening for mutants that accumulated the 67-kD form of CPY that lacked outer chain mannose residues indicative of arrival in the Golgi complex (Figure 3). Mutant cells were radiolabeled at the nonpermissive temperature and spheroplast lysates were immunoprecipitated with antibody to CPY. To detect addition of outer chain mannoses, the radiolabeled CPY was precipitated either with anti-CPY (Figure 3A) or with anti-α1,6-Mannose (Figure 3B) sera. Wild-type cells and sec18 mutant cells, which are blocked in ER-Golgi transport, were included as controls. The mature form of CPY observed in wild-type cells (Figure 3A, lane 1) contained outer chain carbohydrates and was precipitated with anti-α1,6-Mannose serum (Figure 3B, lane 1). In contrast, the p1 form of CPY accumulated in sec18 mutant cells (Figure 3A, lane 2), lacked outer chain carbohydrates and was not precipitated with anti-α1,6-Mannose serum (Figure 3B, lane 2). Six mutants, which defined five complementation groups (SEC31–SEC35), were found to accumulate a form of CPY that comigrated with the p1 form of CPY accumulated in sec18 mutants (Figure 3A, lanes 3–7). The remaining 51 mutants either accumulated other precursor forms of CPY or mature CPY indicating that they were blocked elsewhere in the pathway or were unaffected in CPY transport (data not shown). The form of CPY accumulated in the sec31–sec35 mutants could not be precipitated with the anti-α1,6-Mannose serum and thus lacked outer chain carbohydrate modifications (Figure 3B, lanes 3–7). Also, CPY could not be precipitated from the media/periplasm fraction of the sec31–sec35 mutants confirming that CPY was not secreted aberrantly (data not shown). These results indicated that the sec31–sec35 mutants were blocked in the transport of proteins from the ER to the Golgi complex.

**Transport of soluble and membrane proteins is blocked in the sec31–sec35 mutants:** The severity and inducibility in the secretory block in the sec31–sec35 mutants was examined by measuring the amount of the soluble protein invertase accumulated upon shift to the nonpermissive temperature (Figure 4). Wild-type cells, a restrictive mutant (sec18-1), and a less restrictive mutant (sec21-1) were included as controls. Most of the mutants secreted invertase at nearly wild-type levels at 24°. However, upon shifting to the nonpermissive temperature, the mutants accumulated intracellular in-
invertase, indicating that a block in the secretion of this protein was induced. The \textit{sec32-1} mutant exhibited a particularly restrictive and inducible block in invertase secretion, comparable to that observed for \textit{sec18-1}. The \textit{ypt1-3} allele isolated in this screen also showed a restrictive block in invertase secretion. Less restrictive blocks in invertase secretion, comparable to that of \textit{sec21-1}, were observed in \textit{sec31-1} and \textit{sec35-1} mutants. The block in invertase secretion was nearly identical for \textit{sec34-1} and \textit{sec34-2}; and the \textit{sec33-1} mutant was deficient for invertase secretion at both temperatures.

The effect of the \textit{sec31-sec35} mutations on trafficking of integral membrane proteins was assessed by monitoring the transport of the vacuolar membrane protein, DPAP B (Figure 5). DPAP B is an integral membrane protein that is localized to the vacuole through the early stages of the secretory pathway (ROBERTS \textit{et al.} 1989). The mature form of DPAP B was observed in wild-type cells (lane 1). A precursor form of DPAP B that migrated with a faster mobility than the mature form accumulated in the \textit{sec18-1} mutant (lane 2) and the \textit{sec31-sec35} mutants (lanes 3–7). These results indicated that \textit{sec31-sec35} mutations effectively blocked the transport of both membrane proteins and soluble proteins through the secretory pathway.

Membranes and vesicles accumulated in \textit{sec} mutants:

The \textit{sec} mutants were examined by electron microscopy to assess morphological changes in the membrane compartments of the secretory pathway (Figure 6). We found that various membrane structures were accumulated in the \textit{sec} mutants that were present to a lesser degree in wild-type cells (Figure 6A). As had been observed previously for mutations such as \textit{sec12-4} (Figure 6C) that block the transport of proteins from the ER (NOVICK \textit{et al.} 1980), several of the mutants isolated in our screen exhibited an extensive proliferation of ER membrane including \textit{sec23-5} and \textit{sec33-1}. However, this classic morphology was not observed in all of the mutants blocked in transport of proteins from the ER. The phenotype of the \textit{sec32-1} mutant (Figure 6B) was particularly intriguing. Although the \textit{sec32-1} mutant was very restrictive both for growth and for secretion of invertase at the nonpermissive temperature, this mutant clearly did not accumulate the level of membranes characteristic of the mutants previously classified as defective in transport from the ER. We considered that some ultrastructural features of \textit{sec32-1} may not have been readily apparent in the examination of random cross sections that was used to assess vesicle density and membrane accumulation. We therefore employed limited serial cross section analysis to explore the nature of the membrane structures of the \textit{sec32-1} mutant in greater detail (Figure 7). Indeed, \textit{sec32-1} mutant cells exhibited a distinctive structure, a network of anastomosed membranous tubules with mesh spacing of 80–150 nm. These tubular networks often were seen close to the nucleus and connections to the nuclear membrane were observed (Figure 7, large arrowheads). When tubular network structures were found elsewhere in the cytoplasm, it was more difficult to trace their connections. Once clear views of this tubular network structure were obtained from serial sections, it was possible to identify these structures in random nonserial sections. The tubular network structure was observed in nearly half the \textit{sec32-1} mutant cells. These tubular network structures also were seen occasionally in wild-type cells that indicated that they were not aberrantly formed.
Yeast ER-to-Golgi Mutants

Figure 6.—sec12 sec32 double mutants exhibited a morphology similar to the parental sec12 mutant. Yeast cells were grown in YPD medium at 24°C, then shifted to 38°C for 3 hr. Cells were prepared for electron microscopy using permanganate fixation. (A) Wild type (RSY955), (B) sec32 (RSY955), (C) sec12 (RSY955), (D) sec12 sec32 (RSY996). Bar, 1 μm.

and likely corresponded to normal structures of the secretory pathway.

The density of 50-nm vesicles has been used in earlier studies (Kaiser and Schekman 1990) to group sec mutants blocked in the transport of proteins from the ER into two phenotypic classes: the class I mutants contain a low density of vesicles similar to that of wild-type cells, and the class II mutants contain three to five times the number of vesicles as wild-type cells. We quantified the density of vesicles in wild-type and sec mutant strains isolated in this screen (Table 3). A low density of vesicles, comparable to that of wild type, was observed in sec31-1 and sec23-5 mutants. A high density of vesicles, comparable to that of the sec18-1 mutant, was observed in sec34-1 and sec35-1 mutants. The remaining mutants, sec32-1, sec33-1, and ypt1-3 accumulated a moderate number of vesicles.

The level of membrane accumulation in wild-type and sec mutant strains also was assessed by comparing the length of intracellular membrane to the cell circumference in random cross sections. Wild-type cells (SEC) and two previously isolated ER-blocked secretory mutants (sec12-4 and sec18-1) were included as controls. We found that the sec mutants varied in the degree to which they accumulated membranes (Figure 8). The sec33-1 and sec23-5 mutants showed a high level of membrane accumulation, slightly less than that observed for the sec12-4 mutant. A moderate level of membrane accumulation, comparable to that seen in the sec18-1 mutant, was observed in sec31-1, sec35-1, and ypt1-3 mutants. A slight increase in membrane accumulation relative to wild-type cells was observed in the sec32-1 mutant, while no significant increase was noted in the sec34-1 mutant.

Test of epistasis: The epistatic relationships between
FIGURE 7.—Serial sections of sec32 showed an extensive network of anastomosed membranous tubules. A tubular network structure located close to the nucleus showed several connections to the nuclear envelope (large arrowheads). Other small tubular network structures were located away from the nucleus (small arrowheads). At the cell periphery, the constant cross-sectional view of the ER throughout the serial sections (*) indicated a sheet-like conformation of the membrane. Another membrane structure, possibly a single Golgi cisterna, had several vesicles nearby (arrow). Bar, 0.5 μm.

the morphological phenotype observed in the sec32-1 mutant and the phenotypes of other secretory mutants were determined by double mutant analysis. The sec32-1 mutant was particularly well suited for this analysis as this mutant exhibited a very restrictive block both for growth and invertase secretion upon shift to the non-permissive temperature. Whereas sec32-1 mutants exhibited tubular network structures (Figures 6B and 8), sec12-4 mutants showed an extensive proliferation of ER membrane (Figure 6C), and sec7-5 mutants accumulated stacked membranes. Previous studies have established that mutants that displayed the ER-accumulating phenotype were epistatic to those that displayed the stacked membrane-accumulating phenotype (Novick et al. 1981). We found that the sec12 sec32 double mutant exhibited a phenotype similar to that observed in the sec12-4 parental strain; these double mutants showed an extensive proliferation of ER membrane (Figures 6, C and D, and 7). Double mutants constructed from sec32-1 and sec7-5 resembled the sec32-1 parental strain; these mutants exhibited tubular network structures but lacked stacked membranes. Thus, mutants that displayed the ER-accumulating phenotype were epistatic to those that displayed the tubular network-accumulating phenotype, and mutants that displayed the tubular network-accumulating phenotype were epistatic to those that displayed the stacked membrane-accumulating phenotype.

Identification of genes corresponding to SEC32 and SEC33: Several genes implicated in ER-Golgi transport, including BOS1, SAR1, SED5, and SLY1, had been identified by high copy suppression approaches. To test whether SEC31–SEC35 corresponded to any of these genes, the sec31–sec35 mutants were transformed with plasmids encoding Sarlp, Sed5p, Sly1p, Bos1p, or control plasmids and the growth of transformants at permissive and restrictive temperatures was examined. No suppression of the temperature-sensitive growth phenotype of sec31–sec35 by the SAR1, SED5 and SLY1 genes was observed, even when introduced on high copy plasmids. By contrast, the temperature-sensitive growth defect of sec32-1, but none of the other mutants, was fully suppressed at 37°C by BOS1 on a single-copy plasmid. These results suggested that the gene defective in sec32-1 may be BOS1, and tetrad analysis was performed to test this hypothesis (see MATERIALS AND METHODS). In

<table>
<thead>
<tr>
<th>Strain group</th>
<th>Vesicles per μm² of cell volume</th>
</tr>
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<tr>
<td>Previously isolated mutations</td>
<td>SEC</td>
</tr>
<tr>
<td>RSY255</td>
<td>sec12-1</td>
</tr>
<tr>
<td>RSY263</td>
<td>sec18-1</td>
</tr>
<tr>
<td>Mutations isolated in this study</td>
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</tr>
<tr>
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</tr>
<tr>
<td>RSY977</td>
<td>ypt1-3</td>
</tr>
</tbody>
</table>
The amount of membrane in wild-type and sec mutants strains was assessed by determining the length of intracellular membrane relative to the cell circumference in electron micrographs of permanganate-fixed yeast cells. For each yeast strain analyzed, 60 random cell sections were scored according to five levels of intracellular membrane:cell circumference ratio as follows: level 1 = 0.50, level 2 = 0.50–0.75, level 3 = 0.75–1.00, level 4 = 1.00–1.25, level 5 = >1.25. As compared to SEC cells (RSY855), a substantial increase in membrane was observed in sec12 (RSY263) and sec33 (RSY957) cells. Moderate amounts of membrane were seen in sec18 (RSY271), sec31 (RSY953), and sec35 (RSY963) mutants. A slight increase was observed in sec32 (RSY8955), and no significant increase was observed in sec34 (RSY959) mutants. sec12 sec32 double mutants (RSY996) exhibited membrane accumulation levels comparable to that of the parental sec12 mutant.

A total of 49 tetrads analyzed, only parental ditypes were observed demonstrating that the sec32 and BOS1 loci were closely linked and most likely identical.

We noticed that sec21-1 and sec33-1 displayed strong synthetic lethality, the pattern of spore viability in 33 tetrads analyzed suggested that the double mutant was inviable at 24°. Yet, no synthetic lethality was observed between sec21-1 and sec31-1, sec34-1 or sec35-1 mutants. Since sec21 encodes the γ subunit of coatomer (Hosobuchi et al. 1992), we decided to test whether sec33 might also encode a coatomer subunit. sec33-1 was transformed with plasmids harboring the RET1, SEC21, SEC26 and SEC27 genes, encoding α-, γ-, β- and β' coatomer subunits, respectively, or control plasmids and growth of the transformants was analyzed. A single-copy plasmid harboring the RET1 gene, but not the other three genes, suppressed the temperature-sensitive growth of sec33-1, indicating that SEC33 may be allelic to RET1. To test this possibility, haploid strains carrying the sec33-1 and ret1-1 mutations were crossed. The resulting diploids exhibited temperature-sensitive growth, indicating that the two mutations fall into the same complementation group. Sporulation of the sec33-1 ret1-1 diploid and dissection of 44 tetrads yielded only temperature-sensitive spores, demonstrating that the two loci are closely linked. Thus, the sec33 locus likely is identical to the RET1 locus encoding the α subunit of coatomer.

**DISCUSSION**

**Screen for isolation of sec mutants:** A complete understanding of the secretary pathway in eukaryotic cells will require the identification of all the genes that function in this pathway. Toward this end, we have developed a screen for the isolation of conditional yeast mutants affected in the transport and sorting of secretory proteins. This screen was based on the detection of unprocessed α-factor precursor in yeast colonies lysed on nitrocellulose filters. The ease and expediency of the colony immunoblotting technique made it a powerful tool for screening large numbers of colonies. Mutations in genes acting both early and late in the secretary pathway (Figure 1), as well as mutations affecting vacuolar protein sorting, were readily detected. Since only a single mutant allele was isolated for many of these genes, it is likely that mutations in additional genes that function in the transport and sorting of secretory proteins would be isolated by repeated application of this screen.

**ER-Golgi-blocked sec mutants:** We have focused on the characterization of novel temperature-sensitive mutants blocked in ER-Golgi transport that define five complementation groups, SEC31–SEC35. Like previously isolated ER-Golgi mutants, the sec31–sec35 mutants accumulated ER-modified forms of both soluble and membrane proteins (Figures 3–5) and exhibited alterations in the morphology and quantity of membranes (Figures 6–8) and vesicles (Table 3). The sec mutants differed in levels of membrane accumulation and vesicle density. In some cases, the membrane accumulation and vesicle density were inversely correlated. For example, the sec34-1 mutant showed a high vesicle density and a low level of membrane accumulation, while the sec23-5 mutant showed a low vesicle density and a high level of membrane accumulation. However, this inverse correlation was not always observed. High vesicle densities were present in the sec18-1 and sec34-1 mutants, yet these mutants showed different levels of membrane accumulation. Similarly, the sec23-5 and sec31-1 mutants exhibited comparably low vesicle densities yet differed significantly in membrane accumulation levels. The variations noted in membrane accumulation and vesicle density between the sec mutants may be due to differences in the restrictiveness of the mutant alleles or to membrane structures not easily discerned or quantified by the methods used here. A more
interesting possibility to explain these variations, however, is that secretory protein transport and membrane proliferation may be uncoupled. In this regard it has been observed that yeast cells containing elaborated membrane structures, e.g., around the nucleus (karmellae) do not necessarily exhibit profound defects in secretion or cell growth (WRIGHT et al. 1988).

**SEC31 is involved in vesicle formation:** The low vesicle density observed in the two mutants, sec23-5 and sec31-1, is consistent with the previous designation of sec23-1 as a class I mutation that affects the formation of ER-derived vesicles (KAISER and SCHERMAN 1990) and suggested that sec31-1 also may be defective in this transport step. Biochemical studies have shown that the formation of ER-derived vesicles requires at least three purified protein fractions: Sar1p, Sec23p/Sec24p complex, and Sec13p/p150 complex (SALAMA et al. 1993). These proteins cooperate on ER membranes to form an electron-dense coat, designated COP II, around ER-derived vesicles (BARLOWE et al. 1994). Our lab found that a genomic DNA clone encoding p150 fully suppressed the temperature-sensitive phenotype of sec31-1, and standard genetic methods confirmed that the cloned DNA corresponds to the sec31 locus (N. SALAMA, J. CHUANG and R. SCHERMAN, unpublished results).

Thus, the SEC31 gene product is the p150 subunit of the COP II membrane coat, involved in the formation of ER-derived vesicles. The sec31-1 mutant should prove useful for further study of the function of this protein.

**SEC32 is allelic to BOS1:** Unlike the other ER-Golgi mutants, the sec32-1 mutant did not show an extensive proliferation of ER membrane and showed only a moderate vesicle density. The intermediate number of vesicles displayed by sec32-1 cannot be attributed to leakiness of the allele because sec32-1, like ypl1-3, was very restrictive for both invertase secretion and growth. Surprisingly, sec32-1 mutant cells exhibited a network of membranous tubules (Figure 7), which was unlike the ER-acculminating phenotype of sec12-4 or the stacked membrane phenotype of sec7-5. Double mutant analysis indicated that sec12-4 was epistatic to sec32-1, and that sec32-1 was epistatic to sec7-5. Although the precise nature of these membrane tubules remains unclear, they appear to be morphologically distinct from ER membrane (PREUSS et al. 1991) and Golgi cisternae (PREUSS et al. 1992). Our genetic analysis has identified sec32-1 as the first temperature-sensitive mutation in the BOS1 gene to be reported. BOS1 was originally isolated as a high copy suppressor of the bet1-1 mutation (NEWMAN et al. 1990). BOS1, BET1/SLY12 and SEC22/SLY2 all encode membrane proteins thought to be involved in the fidelity of targeting of ER-derived vesicles to the Golgi complex (reviewed in LIAN and FERRO-NOVICK et al. 1993; ROTHMAN 1994). One possibility is that the sec32-1 mutant forms vesicles that are competent for fusion but, due to inactivation of Bos1p, lack the targeting information that enables them to fuse specifically. If this were the case, then vesicles may fuse with each other or with other membrane organelles resulting in a low or moderate vesicle density and the appearance of new membrane structures and/or an enhancement in existing membrane structure. Further analysis will be necessary to ascertain the function and identity of these novel membrane structures accumulated in sec32-1 cells.

**SEC33 is allelic to RET1, encoding the α subunit of coatomer:** Coatomer is the cytosolic assembly unit of the nonclathrin coat that in mammalian cells is associated with the Golgi complex and Golgi-derived transport vesicles (reviewed in ROTHMAN 1994). In both yeast and mammalian cells, coatomer is a 700- to 800-kD protein complex consisting of seven stoechiometric subunits, designated α, β, β′, γ, δ, ε, and ζ-COP (WATTERS et al. 1991; HOSOBUCHI et al. 1992). Several of the yeast genes encoding subunits of coatomer have been identified, including RET1 (α), SEC26 (β), SEC27 (β′) and SEC21 (γ). Coatomer has been shown to interact specifically with the KXXX-motif present on ER-resident membrane proteins (GOSSON and LETOURNEUR 1994) that is required for the selective retrieval of escaped ER proteins from the Golgi to the ER (GAYNOR et al. 1994). A temperature-sensitive mutation in the RET1 gene, ret1-1, was isolated in a genetic screen for mutants defective in KXXX-retrieval (LETOURREUR et al. 1994). The ret1-1 mutant displays no secretion defect at nonpermissive temperatures, although coatomer from ret1-1 mutants incubated at nonpermissive temperatures has lost the ability to bind KXXX-motifs (LETOURREUR et al. 1994).

Our genetic data demonstrated that sec33-1 was a temperature-sensitive allele of the RET1 gene that showed strong synthetic lethality with sec21-1. sec33-1 accumulated a moderate number of vesicles at the nonpermissive temperature (Table 3) and thus can be placed into the “intermediate” class of mutants defined by KAISER and SCHERMAN (1990), which also includes the coatomer subunit mutants sec21-1 and sec27-1. In contrast to what has been reported for the first mutation isolated in RET1, ret1-1 (LETOURREUR et al. 1994), we found that sec33-1 exhibited a clear secretion phenotype. It has been speculated that coatomer may act only in retrograde Golgi-to-ER traffic, and that its involvement in ER-to-Golgi transport may be indirect (PELHAM 1994). Another possibility, suggested by the phenotypes of the sec33-1 and ret1-1 mutants, is that the α subunit of coatomer may have a role both in forward transport as well as retrograde Golgi-to-ER traffic. Further investigation into the apparent phenotypic differences of the mutant alleles of RET1 may yield additional insight into the function of coatomer in protein trafficking.

**SEC34 and SEC35 may define components of the targeting/fusion machinery:** The high vesicle densities observed in the sec34-1 and sec35-1 mutants suggests that these mutants may be similar to the class II mutations (KAISER and SCHERMAN 1990) with defects in the tar-
targeting or fusion of ER-derived vesicles with the Golgi complex. Our genetic analysis has shown that SEC34 and SEC35 did not correspond to any of the genes known to be involved in the targeting/fusion process, including SEC17, SEC18, BET1/SLY12, SEC22/SLY2, BOS1, USO1, YPT1, SED5, and SLY1, and thus may encode novel proteins involved at this stage of the secretory pathway. Analysis of sec34-1 and sec35-1 mutant phenotypes in vitro assays, cloning of the genes, and biochemical characterization of the gene products will be necessary to define the role of SEC34 and SEC35.

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