Yeast Secretory Mutants That Block the Formation of Active Cell Surface Enzymes

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ABSTRACT   Yeast cells secrete a variety of glycosylated proteins. At least two of these proteins, invertase and acid phosphatase, fail to be secreted in a new class of mutants that are temperature-sensitive for growth. Unlike the yeast secretory mutants previously described (class A sec mutants; Novick, P., C. Field, and R. Schekman, 1980, *Cell.*, 21:205–420), class B sec mutants (sec53, sec59) fail to produce active secretory enzymes at the restrictive temperature (37°C). sec53 and sec59 appear to be defective in reactions associated with the endoplasmic reticulum. Although protein synthesis continues at a nearly normal rate for 2 h at 37°C, incorporation of [³H]mannose into glycoprotein is reduced. Immunoreactive polypeptide forms of invertase accumulate within the cell which have mobilities on SDS PAGE consistent with incomplete glycosylation: sec53 produces little or no glycosylated invertase, and sec59 accumulates forms containing 0–3 of the 9–10 N-linked oligosaccharide chains that are normally added to the protein. In addition to secreted enzymes, maturation of the vacuolar glycoprotein carboxypeptidase Y, incorporation of the plasma membrane sulfate permease activity, and secretion of the major cell wall proteins are blocked at 37°C.

Studies on the secretory process in both procaryotic and eucaryotic cells have focused on the events associated with synthesis and processing of secretory proteins. A number of proposals have been made that seek to explain how proteins pass into or through membranes (1–4). Two of these theories, the signal hypothesis (1) and the membrane trigger hypothesis (2), have different experimental predictions. The signal hypothesis, which was first proposed for secretory proteins and later extended to membrane proteins (5), postulates that an amino terminal signal directs the nascent secretory protein to a site on the endoplasmic reticulum (ER).[1] The signal peptide is cleaved as the secretory protein penetrates into the lumen of the ER. The membrane trigger hypothesis, on the other hand, proposes that the signal sequence allows a precursor to assume a conformation compatible with membrane penetration. According to this view, no membrane proteins are

The following two papers are dedicated to Dr. Arthur Kornberg on his 65th birthday.
[1] *Abbreviations used in this paper:* ConA, concanavalin A; CPY, carboxypeptidase Y; endo H, endoglycosidase H; SRP, signal recognition particle; TNBS, trinitrobenzenesulfonic acid; TNP, trinitrophenol; YP medium, yeast extract-peptone.

needed to facilitate translocation of newly synthesized membrane or secretory proteins. Thus, a fundamental distinction between the two proposals is the requirement for a translocation system.

Recently, a ribonucleoprotein complex (signal recognition particle, SRP) has been shown to bind to polysomes engaged in secretory protein synthesis causing an arrest in translation (6, 7). A 72-kdalton ER protein (the docking protein or the SRP receptor) releases the translation block (8, 9). It has been proposed that the SRP binds to the signal sequence portion of a nascent secretory polypeptide and directs the translation complex to the docking protein on the ER. The SRP and docking protein fulfill functions required at an early step in protein translocation; later steps may require additional functions. Conditional lethal mutants that affect protein translocation could reveal new steps in the penetration process.

Earlier reports from this laboratory have described the isolation and characterization of yeast mutants that are temperature-sensitive for growth and secretion (10, 11). These mutants (class A *sec* mutants) fail to secrete invertase and acid phosphatase at the restrictive temperature (37°C), and instead accumulate active enzymes within membrane-en-

closed organelles. The class A *sec* mutants are able to translocate proteins across the ER but these are blocked at various stages of transport to the cell surface.

Glycoproteins acquire N-glycosidically linked oligosaccharides during transit across the ER membrane (12). The transfer of oligosaccharide to protein should be blocked indirectly if polypeptide transit into the ER is disrupted. Mutants that block this transit step should synthesize secretory proteins that contain little or no carbohydrate. Under these conditions certain unglycosylated secretory proteins will not fold into active enzymes. In particular, yeast invertase and acid phosphatase, both of which normally contain about 50% carbohydrate, are not produced in an active form when oligosaccharide synthesis is blocked by treatment of cells with tunicamycin (13, 14). In this report we describe a new class of yeast secretory mutants which appear to affect the transit of secretory, vacuolar, and plasma membrane polypeptides into the ER.

MATERIALS AND METHODS

Strains, Growth Conditions, Reagents, and Buffers: Saccharomyces cerevisiae haploid strain X2180-1A (a, gal2) was obtained from the Yeast Genetics Stock Center (Berkeley, California). BR231-8B (*SUC0*) was obtained from S. Fogel (University of California, Berkeley). pep4-3 was obtained from E. W. Jones (Carnegie Mellon University). pRB58 is a 2 μm plasmid containing the *SUC2* gene (15). All *sec* mutant strains were derived from X2180-1A. Standard genetic techniques were used to construct SF402-4D, SF649-2A/pRB58, SF629-3C/pRB58, and SF610-7B/pRB58. HMSF331 (*sec53*-6) and HMSF375 (*sec53*-11) are original isolates. Standard genetic techniques were used to construct haploid double *sec* mutant strains. Double mutants were evaluated by genetic complementation with both parent strains to ensure retention of both mutant alleles.

YPD medium (yeast extract-peptone-dextrose) contained 1% Bacto-Yeast extract, 2% Bacto-Peptone and 2% glucose; YP medium was the same with different levels of glucose. Wickerham's minimal medium (16) was used with different levels of glucose; for sulfate-free medium, chloride salts replaced all sulfate salts; for labeling with [³H]mannose, minimal medium was supplemented with 0.5% casamino acids, 1.8% galactose, and 0.2% glucose. For strains harboring the pRB58 plasmid, minimal medium was supplemented with histidine (20 μg/ml) and lysine (20 μg/ml). Phosphate-depleted YPD was prepared as described by Rubin (17). To change growth medium, cells were sedimented in a clinical centrifuge and resuspended in new medium. The absorbance of cell suspensions was measured in a 1-cm quartz cuvette at 600 nm in a Zeiss PMQ II spectrophotometer; 1 OD$_{600}$ U corresponds to .15 mg dry wt.

Other reagents were obtained as indicated: glucose oxidase, o-dianisidine, peroxidase, p-nitrophenylphosphate, cycloheximide, phenylmethylsulfonyl fluoride, tosyllysine chloromethyl ketone, tunicamycin, trinitrobenzene sulfonic acid, and concanavalin A (ConA)-Sepharose 4B (8 mg protein/ml gel) were from Sigma Chemical Co. (St. Louis, MO); H₂³⁵SO₄ was from ICN K & K Laboratories, (Plainview, NY) and Amersham (Arlington Heights, IL); Enhance and D-[1-³H]mannose (13.2 Ci/mmole) were from New England Nuclear (Boston, MA); IgG Sorb (fixed *Staphylococcus aureus* Cowan I cells) was from the Enzyme Center (Boston, MA); glutaraldehyde, osmium tetroxide, and spurr embedding medium were from Polysciences, Inc. (Warrington, PA); endoglycosidase H (endo H) was generously provided by P. Robbins (Massachusetts Institute of Technology). ConA-Sepharose was washed four times with an equal volume of 0.5 M NaCl, 0.5 M α-methyl-D-mannoside, then twice with reaction buffer (described below), and resuspended in an equal volume of the same buffer. Lyticase is a yeast lytic enzyme preparation useful in spheroplast formation (18). Fraction II (30,000 U/mg; 1 U will lyse 0.2 OD$_{600}$ U of logarithmic phase cells in 30 min at 30°C) was used. Affinity-purified anticarboxypeptidase Y IgG, provided by T. Stevens (this laboratory), was prepared as described elsewhere (19). Invertase antiserum was prepared as described by Esmon et al. (20). Specific antibody was obtained in a two-step procedure. Antiserum was treated first with intact *SUC0* cells to remove antibody against carbohydrate; antibody directed against the protein portion was then affinity purified by absorption and elution from invertase conjugated to Sepharose 4B.

PBS is 0.2 M NaCl, 12.5 mM potassium phosphate (pH 7.6). Spheroplasting medium is 1.4 M sorbitol, 25 mM potassium (or sodium phosphate (pH 7.5), 2 mM MgCl₂, 10 mM sodium azide, 40 mM β-mercaptoethanol, and 50 U of lyticase per OD$_{600}$ U of cells. Dilution buffer is PBS with 2% Triton X-100. Reaction buffer is 2.5% Triton X-100, 0.5 M NaCl, 1 mM magnesium chloride, 20 mM triethanolamine·HCl (pH 7.5). Wash buffer is 0.5 M NaCl, 0.05% SDS, 20 mM triethanolamine·HCl (pH 7.5).

Isolation of Secretory (sec) Mutants: Mutants were isolated by the density enrichment procedure of Novick et al. (11). Strains that failed to accumulate or secrete active invertase upon derepression at 37°C were put into complementation groups by standard genetic techniques. A member from each group that showed minimal secretion of invertase at 37°C was analyzed for protein synthesis by measuring the incorporation of ³⁵SO₄²⁻ into acid-insoluble material (10). Strains that synthesized protein at ~50% or more of the wild-type (X2180) rate at 37°C were candidates for secretory mutants.

Immune Precipitation and Electrophoresis: For invertase immunoprecipitation, cells were grown to early exponential phase in minimal medium supplemented with 100 μM (NH₄)₂SO₄ and 5% glucose. Aliquots (~2 OD$_{600}$ U) were incubated at 24° or 37°C for 30 min, sedimented in a table top clinical centrifuge, and resuspended in 0.6-1 ml of medium containing 25-50 μM (NH₄)₂SO₄ and 0.1% glucose. The incubation was continued at the same temperature for 30 min to 1 h in the presence of 400-750 μCi of ³⁵SO₄²⁻. These conditions allow efficient radiolabeling of invertase which is derepressed in medium containing low glucose. Labeled cells were centrifuged and washed once with cold 10 mM sodium azide. Tunicamycin (5-10 μg/ml), when used, was included during the 30 min incubation prior to the change in medium, and during the subsequent labeling period.

Cell lysates were prepared by one of two lysis procedures; rapid lysis with glass beads or conversion to spheroplasts with lyticase. In the rapid lysis procedure, cell pellets were mixed with 0.15 g glass beads (0.3-0.5 mm) and 30 μl of 1% SDS, broken by being vortexed for 90 s at 4°C, and then immediately heated in a boiling water bath for 3 min. Lysates were diluted with 0.8 ml of dilution buffer and centrifuged at 27,000 g for 20 min. Aliquots (0.7 ml) of each sample were treated with 1 μl of affinity-purified antibody (2.9 mg/ml) and incubated at 0°C for 16-18 h. In the enzymatic lysis procedure, cells were resuspended in 0.2 ml of spheroplasting medium and incubated for 60 min at 30°C. The spheroplast supernatant and pellet fractions were separated by sedimentation at 3,000 g for 10 min. The pellet was resuspended in 0.1 ml of 1% Triton X-100, or in 50 mM sodium phosphate (pH 7.5), or solubilized by heating in 1% SDS. Samples lysed in Triton X-100 were diluted with an equal volume of 2× PBS containing 2 mM each of phenylmethylsulfonyl fluoride and tosyllysine chloromethyl ketone. Samples lysed with SDS were diluted with 0.8 ml of dilution buffer. All lysates were centrifuged at 27,000 g for 20 min. The spheroplast supernatant fraction was adjusted to 0.6 ml with PBS, Triton X-100, phenylmethylsulfonyl fluoride, and tosyllysine chloromethyl ketone (final concentrations 0.66×, 1%, 1 mM, and 1 mM, respectively), and centrifuged at 100,000 g for 90 min. The final supernatant fractions were mixed with 1-6 μl of affinity-purified invertase antibody (2.9 mg/ml) in Eppendorf tubes and incubated at 0°C for 16-18 h. Mixtures were treated with fixed *S. aureus* cells (75 μl of a 10% [wt/vol] suspension was used for spheroplast supernatant fractions and 25 μl for spheroplast lysates) and the immune precipitates were washed as described elsewhere (21). In a control immune competition experiment, twice the number of cells were used and the lysate was split; antibody preincubated with 10 μg of purified invertase was added to one-half of the fraction. Protein bands that were competed with purified invertase were not competed with bulk mannan isolated from an invertase mutant strain (not shown), indicating that the antibody was directed against the protein and not the carbohydrate portion of invertase. Final pellets were resuspended in a volume of SDS gel sample buffer that was proportional to the incorporation of ³⁵SO₄²⁻ 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, fixed *S. aureus* cells were sedimented, and the supernatant samples were transferred to new tubes. Electrophoresis was performed by the Laemmli procedure (22) or, for 10% polyacrylamide SDS slab gels (13.7 × 16.7 cm), a modified procedure was used (23). Molecular mass standards were: RNA polymerase (α-subunit, 39 kdalton; β-subunit, 155 kdalton; β' subunit, 165 kdalton), phosphorylase a (94 kdalton), BSA (68 kdalton), ovalbumin (43 kdalton), carbonic anhydrase (30 kdalton), and trypsin inhibitor (21.5 kdalton). Gels were stained for protein and treated with Enhance according to the procedure described by New England Nuclear.

Glycosylated forms of invertase were evaluated by treatment with endo H as described by Esmon et al. (20), or by binding to ConA-Sepharose 4B. In the latter procedure, radiolabeled cells were converted to spheroplasts which were lysed with 1% Triton X-100 as described above. The Triton-insoluble fraction was resuspended in 25 μl of 1% SDS, heated in a boiling water bath for 3 min, and diluted with 0.4 ml of reaction buffer. After centrifugation for 30 min at 27,000 g, half of the soluble fraction was mixed with ConA-Sepharose (40 μl) and agitated for 3 h at 24°C. The ConA-Sepharose was then sedimented; the supernatant fraction was set aside and the pellet fraction was washed three

times with 0.5 ml of wash buffer, resuspended in 50 μl of 1% SDS, and heated for 3 min in a boiling water bath. Dilution buffer (0.2 ml) was added and the sample was centrifuged for 5 min in an Eppendorf microfuge. Dilution buffer (0.2 ml) was also added to the ConA-Sepharose nonbinding fraction, and to the sample that was not treated with ConA-Sepharose. Invertase was immunoprecipitated and electrophoresed as above.

For carboxypeptidase Y (CPY) immunoprecipitates, cells were grown in minimal medium supplemented with 50 μM $(NH_4)_2SO_4$ and 2% glucose. The incubation conditions were the same as described above except that cells were incubated for 1 h at 24°C, or 37°C in the presence of 200 μCi of $^{35}SO_4^{2-}$, in fresh minimal medium supplemented with 50 μM $(NH_4)_2SO_4$ and 2% glucose. Cell lysates were prepared by the rapid lysis procedure. Supernatant fractions were incubated with 2–4 μl of antibody (1 mg/ml) for 2 h at 0°C. Samples were treated with S. aureus, washed, solubilized, and electrophoresed as described above.

Enzyme Assays and Other Procedures: External invertase was assayed as previously described (24); 1 U is defined as 1 μmol of glucose released per min. Cytoplasmic invertase was assayed in spheroplast lysates as described by Novick and Schekman (10). External acid phosphatase was assayed as described by van Rijn, Boer, and Steyn-Parvé (25); 1 U is defined as 1 nmol of p-nitrophenol released per minute. Sulfate permease was assayed as described by Breton and Surdin-Kerjan (26); 1 U is defined as 1 nmol of SO_4^{2-} taken up per minute. Radioactivity was measured in a Searle Delta 300 liquid scintillation counter. Treatment of yeast cells with trinitrobenzenesulfonic acid (TNBS) and evaluation of trinitrophenol (TNP)-tagged ^{35}S-labeled proteins are described elsewhere (27). Electron microscopy was performed by the method of Byers and Goetsch (28).

RESULTS

Secretory Mutants That Do Not Accumulate Active Invertase

The enrichment procedure used to isolate yeast secretory mutants requires that cells become dense at 37°C. This technique appears to enrich for cells that continue macromolecular synthesis without a corresponding increase in cell size or number (11). Of the original 485 isolates that failed to secrete invertase and acid phosphatase, only 190 accumulated active invertase at 37°C; these were called class A *sec* mutants. Standard genetic techniques were used to arrange the remaining mutants into 34 complementation groups. Nearly 70% of the mutants were in two groups, 18 groups had more than one member, and 16 groups had one each. The mutants were then screened for defects in protein synthesis. Only five of the

groups had members that synthesized protein at ~50% or more of the wild-type (X2180-1A) rate at 37°C; these were considered candidates for a new class of secretory mutants.

S. cerevisiae produces two forms of invertase: a constitutive nonglycosylated form that is located in the cytoplasm, and a heavily glycosylated form that is secreted into the cell wall and is under hexose repression (29). The constitutive form is not a precursor of the secreted enzyme (13). In spite of these differences, both enzymes are products of the same gene (15, 30, 31). Secretory mutations should affect the production of active secretory forms of invertase, but not the cytoplasmic enzyme. Candidates for secretory specific defects were grown in repressing medium (YP + 5% glucose) at 24°C. Cells were incubated at 37°C for 30 min before invertase synthesis was derepressed by transfer into YP medium + 0.1% glucose at 37°C for 90 min. External invertase was assayed in whole cells, and intracellular forms of invertase were assayed in spheroplast lysates (10). All five of the *sec* mutant candidates secreted nearly normal levels of invertase at 24°C, but at 37°C showed a threefold or greater difference between the rates (normalized to wild type) of protein synthesis and invertase secretion. Unlike the class A *sec* mutants (*sec1* example in Table I), the new mutants did not accumulate derepressed invertase activity. However, cytoplasmic invertase was produced in four of the five selected mutants when cells were incubated at 37°C in repressing medium. The mutants that synthesized cytoplasmic but not secretory invertase were called the class B *sec* mutants (*sec53*, *sec55*, *sec58*, and *sec59*).

Of the class B *sec* mutants only *sec53* and *sec59* will be considered. Data on the synthesis of secretory and cytoplasmic forms of invertase, and protein synthesis in these mutants in comparison to X2180-1A and *sec1* are presented in Table I. By immunoprecipitation analysis *sec55* and *sec58* produced and secreted immunoreactive, highly glycosylated forms of invertase. We have not determined if the invertase secreted in *sec55* and *sec58* becomes inactive during or after export, or fails to fold correctly during synthesis.

In genetic crosses with the parent strain, mutants of each group were recessive and showed 2:2 segregation of the temperature-sensitive phenotype which always coincided with the

TABLE I

Class B sec Mutants: Secretory and Cytoplasmic Forms of Invertase and Protein Synthesis

Strain	sec	Number of isolates	Derepressed invertase*			Cytoplasmic invertase[‡] (2 h/0 h 37°C)	Protein synthesis[§] (% rate relative to X2180)
			External (90 min 37°C)	Internal (90 min 37°C)	External (90 min 24°C)		
				U/mg dry wt			
X2180-1A (wild type)	—	—	0.36	0.067	0.253	1.63	100
HMSF 331	53-6	16	0.067	0.08	0.267	1.56	50
SF402-4D[‖]	59-1	1	0.047	0.073	0.213	1.68	68
HMSF 1	1-1	—	0.047	1.3	0.207	1.57	—
X2180-1A + tunicamycin (5 μg/ml)	—	—	0.02	0.06	—	1.4	90

* Cells were grown to early exponential phase in YP medium + 5% glucose at 24°C. Samples (2 OD$_{600}$ U of cells) were incubated for 30 min at 37°C, sedimented in a clinical centrifuge, resuspended in 2 ml of YP medium + 0.1% glucose, and incubated for an additional 90 min at 37°C. Parallel samples were incubated at 24°C. At the end of the experiment, cells were sedimented and resuspended in 1 ml of cold 10 mM sodium azide. Cell aliquots (0.5 ml) were sedimented and resuspended in 0.13 ml of spheroplasting medium. Spheroplasts formed after a 60 min incubation at 30°C were sedimented at 3000 g for 10 min and the pellet was resuspended in 0.5 ml of 1% Triton X-100. External invertase was assayed in whole-cell aliquots and internal invertase was measured in the spheroplast lysates.
[‡] Incubations were initiated as in *, except 0 time samples (2 OD$_{600}$ U) were collected and the incubation at 37°C was performed in fresh YP medium + 5% glucose for 2 h. Invertase activity in spheroplast lysates was measured and the unit ratios of the 2 h/0 h samples are presented.
[§] Cells were grown overnight in minimal medium (16) supplemented with 10 μM ammonium sulfate and 2% glucose. Cells (1.5 OD$_{600}$ U) were sedimented and resuspended in 1.5 ml of fresh medium, and incubated at 37°C for 30 min. Label (0.1 μCi of $^{35}SO_4^{2-}$) was added to 1 ml of each sample and the incubation was continued for 90 min; the remaining 0.5 ml was used to normalize the incorporation rate to cell absorbance. Incubations were stopped with 1 ml of cold 20% trichloroacetic acid and incorporation was measured in samples collected onto Whatman GF/A filters (Whatman Laboratory Products Inc., Clifton, NJ).
[‖] SF402-4D was derived from a cross of HMSF 392 (original isolate) and X2180-1A.

secretory defect (data not shown). The class B *sec* mutants were not allelic with any of the class A mutants, and thus represent an independent set of complementation groups.

Immunoreactive Invertase Accumulated in sec53 and sec59

Immunoprecipitation was performed to test the possibility that enzymatically inactive forms of invertase are produced but not secreted in the class B mutants. The analysis was aided by the use of a high copy number plasmid that contains the invertase gene (*SUC2*) on an insert (15). Mutant and wild-type transformed cells were derepressed and radiolabeled with $^{35}SO_4^{2-}$ for 30 min. Immunoreactive forms of invertase were examined in mutant cells that had been converted to spheroplasts with lyticase (10, 18). In this procedure, secreted proteins are released, and accumulated proteins are retained within the spheroplast. Derepressed invertase cross-reactive material was detected in spheroplast lysates from cells labeled at 37°C but not at 24°C (Fig. 1, lanes *1–5*). *sec53* accumulated heterogeneous forms ranging from 60–64.5 kdalton (Fig. 1, lane *1*), while *sec59* produced several closely migrating protein species ranging from 60 to 66 kdalton (Fig. 1, lane *3*). A 60-kdalton species, representing cytoplasmic invertase (32), was detected in spheroplasts from cells labeled at 24°C (Fig. 1, lanes *2* and *4*). At 24°C both *sec53* and *sec59* secreted active invertase that had an electrophoretic mobility similar to the wild-type enzyme (Fig. 1, lanes *6–8*). At 37°C only the spheroplast supernatant fraction from wild-type cells contained
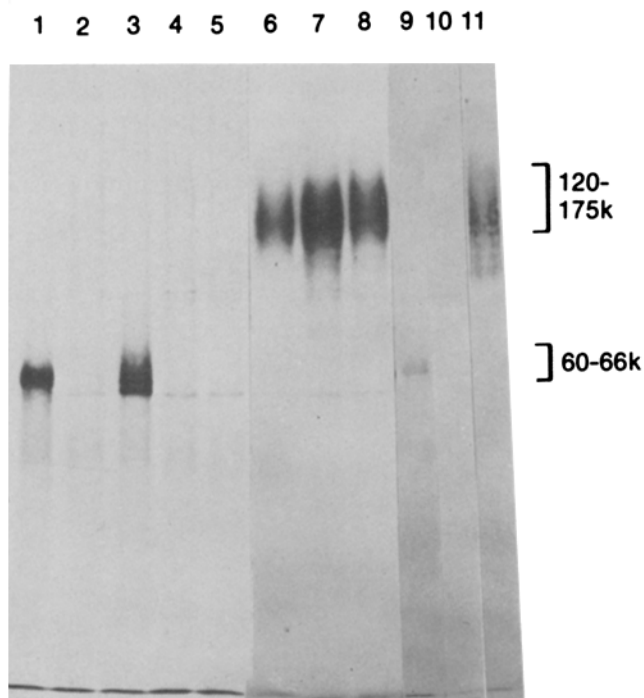

FIGURE 1   Immunoprecipitation of accumulated and secreted invertase in *sec53* and *sec59* at 37° and 24°C. Cell lysates were prepared by the enzymatic lysis procedure. Samples were electrophoresed on a SDS 10% polyacrylamide slab gel (23). A fluorograph of the gel is shown. Spheroplast lysates: (lane *1*) *sec53* (SF649-2A/pRB58) 37°C; (lane *2*) *sec53*, 24°C; (lane *3*) *sec59* (SF629-3C/pRB58) 37°C; (lane *4*) *sec59*, 24°C; (lane *5*) wild type (SF610-7B/pRB58) 37°C. Spheroplast supernatants: (lane *6*) *sec53*, 24°C; (lane *7*) *sec59*, 24°C; (lane *8*) wild type, 24°C; (lane *9*) *sec53*, 37°C; (lane *10*) *sec59*, 37°C; (lane *11*) wild type, 37°C.
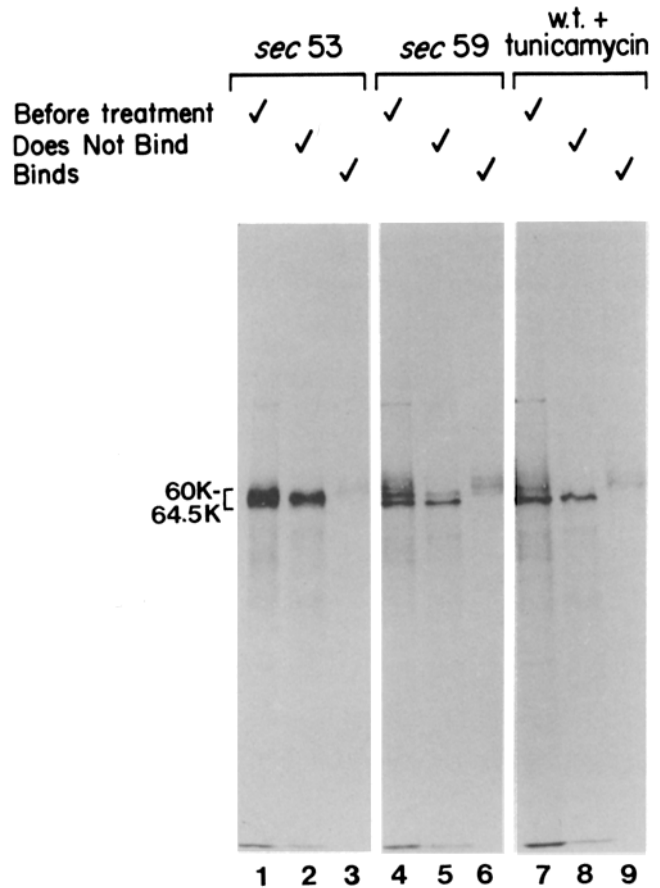
FIGURE 2   Binding of invertase cross-reacting material to ConA-Sepharose. SDS soluble fractions were mixed with ConA-Sepharose. Invertase in untreated, ConA-Sepharose–bound, and -unbound fractions was immunoprecipitated and analyzed by SDS gel electrophoresis. A fluorograph of the gel is shown.

immunoreactive invertase (Fig. 1, lanes *9–11*). Immune precipitation of all species was inhibited effectively by competition with excess unlabeled invertase (data not shown).

The electrophoretic mobility of accumulated invertase precursors suggested partial glycosylation. When transport from the ER is blocked in a class A *sec* mutant, accumulated invertase has a mobility corresponding to ~80 kdalton; ~20 kdalton is accounted for by 9–10 N-linked core oligosaccharides (20, 33). The carbohydrate content of forms accumulated in the class B *sec* mutants was examined by binding to conA and by sensitivity to endo H. Mutant and wild-type cells were labeled with $^{35}SO_4^{2-}$ at 37°C, converted to spheroplasts, and lysed with 1% Triton X-100. The insoluble fraction, which contains the accumulated forms of invertase (see accompanying paper), was dissolved in 1% SDS, and the soluble material was diluted in a Triton-containing buffer and mixed with ConA-Sepharose 4B. Proteins that bound to the immobilized lectin were separated from unbound material by sedimentation. The bound fractions were solubilized again with SDS, and compared with unbound and untreated fractions after immunoprecipitation of invertase. Fig. 2 shows a fluorograph of the species resolved by SDS gel electrophoresis. Most of the invertase in *sec53* did not bind to ConA-Sepharose; the small amount that did bind (~10%) migrated as a species of ~64 kdalton. The multiple species accumulated in *sec59* showed unequal affinity for ConA-Sepharose: forms of 66 and 64.5 kdalton were bound, some of the 63-kdalton

form was bound, and none of the 60-kdalton form was bound. As a control, wild-type cells were treated with tunicamycin, a drug that blocks N-linked oligosaccharide synthesis (34, 35). The 60-kdalton species in tunicamycin-treated cells did not bind to ConA-Sepharose, while a minor form at 63 kdalton, which probably results from incomplete inhibition of oligosaccharide synthesis, was quantitatively bound (Fig. 2).

Endo H cleaves between the two N-acetylglucosamine units that connect N-glycosidically linked oligosaccharides to yeast mannoproteins (36). When invertase from wild-type cells was treated with endo H, a 63-kalton form was produced which represents a 60-kdalton polypeptide with 9–10 remaining GlcNAc residues (Fig. 3). In addition to a 60-kdalton species, an endo H–resistant form of ~63 kdalton persisted in inver-

tase from sec59. Endo H treatment had no effect on the diffuse mobility of the invertase band from sec53. Thus by the ConA-Sepharose and endo H–sensitivity criteria, the invertase in sec53 had very little carbohydrate, while 2–3 of the invertase species accumulated in sec59 were glycosylated.

Other Cell Surface–related Defects in sec53 and sec59

The number of cells and buds remained nearly constant in cultures of mutants incubated for 2 h at 37°C (data not shown). As expected from the enrichment procedure, the halt in bud emergence was accompanied by an increase in cell density (data not shown).

The phenotype of sec53 and sec59 was mimicked to some extent by treatment of wild-type cells with tunicamycin. The drug blocks formation of active secretory invertase without affecting production of the cytoplasmic enzyme (13; Table I); it also caused cells to become dense (data not shown). Thus, the density enrichment may have selected for mutants that failed to synthesize or transfer oligosaccharides to protein. Yeast mannoprotein oligosaccharide synthesis may be measured directly by the incorporation of [³H]mannose into ethanol precipitable material (37). Control experiments showed that synthesis was nearly completely blocked by inhibition of protein synthesis with cycloheximide, while tunicamycin allowed a reduced level of incorporation (Table II). sec53 and sec59 showed a significant reduction in mannose incorporation at 37°C but not at 24°C. The initial rate of [³H]mannose permeation into intact cells was not reduced at 37°C in sec53 (not shown), indicating that mannose transport was not affected.

Two other cell surface markers whose export is blocked in the class A sec mutants (11) were examined in the class B mutants. Acid phosphatase, a cell wall glycoprotein, began to derepress in X2180-1A 2-½ h after a shift from phosphate-rich to phosphate-depleted growth medium. Secretion was measured during the period from 2.5 to 5 h after the medium shift. The sec mutants secreted normally at 24°C, but showed a dramatic reduction in the amount of active acid phosphatase secreted at 37°C. As with invertase, acid phosphatase production was blocked by tunicamycin (Table II). To assess plasma
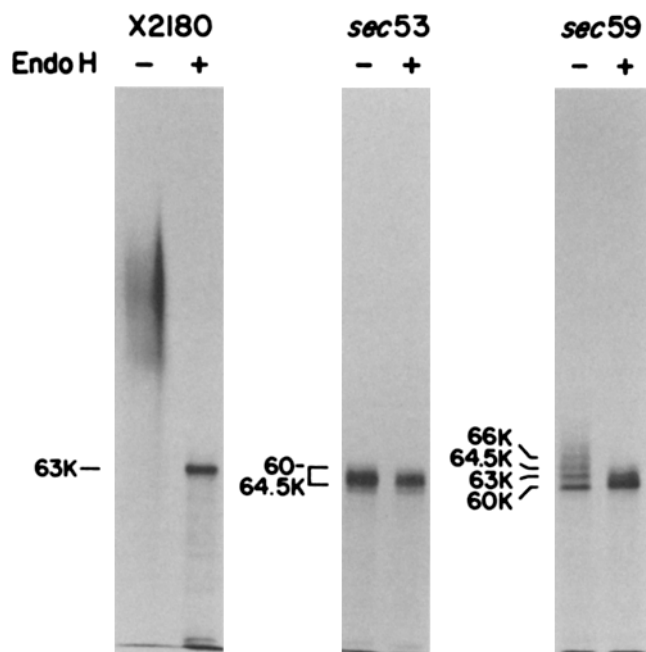

Figure 3   Endo H treatment of immunoreactive invertase. SDS soluble fractions of invertase from wild-type and mutant cells were treated with or without endo H and analyzed by SDS gel electrophoresis. A fluorograph of the gel is shown.

Table II

Oligosaccharide Synthesis and Acid Phosphatase and Sulfate Permease Export in Class B sec Mutants

Strain	sec	Mannose incorporation* (% rate relative to X2180)		Acid phosphatase[‡]			Sulfate permease[§]	
		24°C	37°C	2.5 h	5 h (37°C)	5 h (24°C)	24°C	37°C
					U/ml		U/mg dry wt	
X2180-1A		100	100	19	145	165	5.6	3.9
HMSF331	53-6	98	7	23	23	178	5.5	0.16
SF402-4D	59-1	102	29	30	31	143	5.1	0.6
X2180-1A + tunicamycin (5 µg/ml)		—	21	29	37	—	5.6	3.6
X2180-1A + cycloheximide (100 µg/ml)		—	4	—	—	—	—	—

* Cells were grown overnight in minimal medium supplemented with 0.5% casamino acids, 1.8% galactose, and 0.2% glucose. Cells (5.5 OD₆₀₀ U) were sedimented and resuspended in 1.1 ml of fresh medium. The cultures were incubated at 37°C for 30 min. [³H]mannose (2 µCi) was added to 1 ml of each sample and incubated for an additional 90 min at 37°C; the remaining 0.1 ml was used to normalize the incorporation rate to cell absorbance. Parallel incubations were performed at 24°C. Cells were collected and washed with azide. Spheroplasts were prepared as described in the legend to Table I and lysed with water. Mannoproteins were precipitated with ethanol (final concentration 70%). The insoluble material was collected and counted as previously described (20).
‡ Experiment and enzyme assay were performed as previously described (11).
§ Experiment and assay were performed as previously described (11), except starting cultures were grown with 0.1 mM (NH₄)₂SO₄.

membrane assembly, the appearance of an inducible permease activity was analyzed (10). Sulfate permease is derepressed during a 2.5-h incubation in sulfate-free growth medium (10). Although sulfate permease activity appeared in the mutants during derepression at 24°C, it was significantly lower at 37°C (Table II). In contrast to invertase and acid phosphatase, sulfate permease derepression was not affected by treatment of wild-type cells with tunicamycin.

Treatment of cells with TNBS tags surface proteins with TNP groups; precipitation with anti-TNP IgG allows selective recovery of tagged proteins from a spheroplast supernatant fraction (27, 38). Four major secreted proteins are not exported at 37°C in representative class A *sec* mutants (27). The same test was repeated with *sec53* and *sec59*. Cells were incubated at 25° or 37°C for 30 min, labeled with $^{35}SO_4^{2-}$ for 45 min at the same temperature, and tagged with TNBS at 0°C as previously described (27). Treated cells were converted to spheroplasts, and TNP-tagged proteins were immunoprecipitated from the spheroplast supernatant fraction. Aliquots of solubilized precipitates were analyzed by SDS gel electrophoresis and fluorography. Fig. 4 shows that wild-type cells at 37° and 25°C, and *sec* mutant cells at 25°C, secreted the same set of surface proteins: S_1 (>250 kdalton), S_2 (140 kdalton), S_3 (51 kdalton), and S_4 (33 kdalton). Proteins S_1–S_4

FIGURE 5 Immunoprecipitation of carboxypeptidase Y. Samples were electrophoresed on a SDS 7.5% polyacrylamide slab gel. (lane 1) pep4-3 treated with tunicamycin, 37°C; (lane 2) wild type (X2180-1A) treated with tunicamycin, 37°C; (lane 3) sec53 (HMSF 331) 37°C; (lane 4) sec59 (SF402-4D) 37°C; (lane 5) wild-type, 37°C; (lane 6) pep4-3, 37°C.
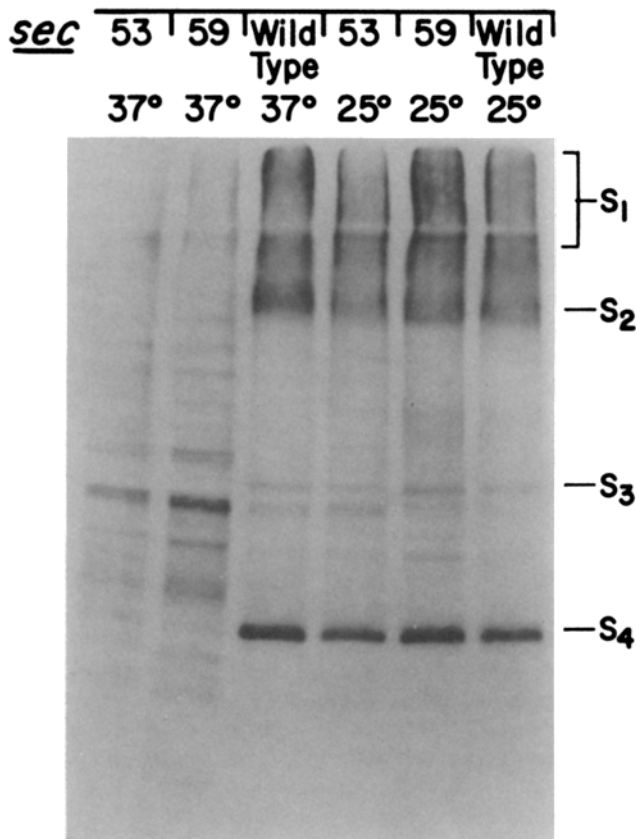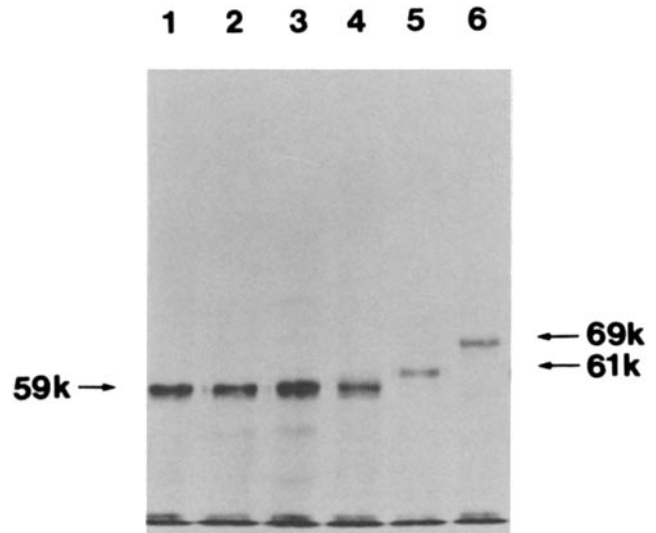

FIGURE 4 Transport of major secreted proteins. Cells were radiolabeled at 37 or 25°C, tagged with TNBS at 0°C, and converted to spheroplasts as previously described (27). Secreted proteins tagged with TNP were immunoprecipitated with TNP antibody and solubilized immunoprecipitates were evaluated by electrophoresis on an SDS 12% polyacrylamide gel. A fluorograph of the gel is presented. (lane 1) sec53-11 (HMSF 375) 37°C; (lane 2) sec59 (SF402-4D) 37°C; (lane 3) wild type (X2180-1A) 37°C; (lane 4) sec53, 25°C; (lane 5) sec59, 25°C; (lane 6) wild type, 25°C.

were not exported at 37°C in the mutants. Instead, immunoprecipitates contained a series of proteins that appeared unrelated to proteins detected under permissive incubation conditions. These proteins, which we suspect are of cytoplasmic origin, were also detected in the cell wall fraction of class A *sec* mutants radiolabeled at 37°C (27). A small and variable percentage of *sec* mutant cells become permeable during incubation at 37°C. Subsequent TNBS treatment allows tagging of intracellular proteins that are released when cells are converted to spheroplasts.

Influence on Vacuolar Glycoprotein Synthesis

The yeast vacuole contains a distinct set of glycoproteins, many of which are hydrolytic enzymes with acid pH optima. One of these, CPY, is transported to the vacuole as a proenzyme with a polypeptide molecular mass of 59 kdalton and N-glycosidically linked oligosaccharides which account for an additional 10 kdalton (39). Localization of the proenzyme requires part of the secretory pathway: class A *sec* mutants that accumulate ER or Golgi bodies block proCPY transport (19). Within the vacuole, proteolytic activation of CPY removes an 8 kdalton amino-terminal propeptide in a reaction that requires the *PEP4* gene product (19, 40).

The effect of the class B *sec* mutants on CPY synthesis and maturation was examined by immunoprecipitation with affinity-purified antibody directed against the CPY protein portion. An immunoreactive form with the mobility of unglycosylated proCPY (59 kdalton) was produced in *sec53* and *sec59* (Fig. 5, lanes 3 and 4) at 37°C, while mature CPY (61 kdalton) was produced at 24°C in these mutants (data not shown). The 59-kdalton form was also produced when wild-type (X2180-1A) or proCPY processing mutant (*pep4*) cells were treated with tunicamycin at 37°C (Fig. 5, lanes 1 and 2); these conditions delay, but do not prevent, transport of unglycosylated proCPY to the vacuole (19). At 37°C in the absence of tunicamycin, wild-type and *pep4* cells produced the expected 61 (Fig. 5, lane 5) and 69-kdalton proCPY (Fig.

Table III
Invertase in Double sec Mutants

Strain	Genotype	Invertase*
		U/mg dry wt
X2180-1A	wild type	0.15
SF 282-2A	sec-18	0.85
SF 502-7A	sec18, sec53	0.24
SF 615-2A	sec18, sec59	0.29

* Cultures were grown in YP + 5% glucose medium at 24°C to an A_{600} of 0.5-1.5. Cells (2 OD_{600} U) were incubated at 37°C for 1 h, sedimented in a clinical centrifuge, and resuspended in 1 ml of YP + 0.1% glucose medium. The incubation was continued for 90 min at 37°C, after which the cells were chilled, sedimented, and resuspended in 10 mM sodium azide. Cells were converted to spheroplasts and accumulated invertase was assayed as before (11).

5, lane 6), respectively; these forms reach the vacuole normally at 37°C (19).

Mutations Block Early in the Secretory Pathway

Yeast secretory mutants that accumulate active secretory enzymes (class A sec) define a pathway of ER → Golgi body → vesicle → cell surface (11, 21). This pathway was determined in part by the demonstration that mutations affecting early stages are epistatic to mutations that block later stages. By this analysis, transport from the ER is the earliest step defined in class A sec mutants. If sec53 (or sec59) blocks a step before the one blocked in sec18 (an ER-accumulating class A mutant), then double sec mutants should fail to accumulate active invertase at 37°C. Alternatively, if sec53 (or sec59) leads to inactivation of invertase at a stage subsequent to the ER, double mutants should accumulate active invertase. Wild-type, sec18, sec18 sec53, and sec18 sec59 strains were derepressed for invertase at 37°C for 90 min. Cells were converted to spheroplasts to release secreted enzymes, and accumulated invertase was assayed in spheroplast lysates. Table III shows that much less invertase was accumulated in the double mutants than in sec18 alone. The level in the double mutants was comparable to the amount found in the wild-type lysate, most of which corresponds to cytoplasmic invertase (32). Thus, sec53 and sec59 are epistatic to sec18.

Thin sections of sec53 and sec59 cells grown at 25° and 37°C were examined in the electron microscope to assess the cytologic consequences of these lesions. Wild-type cells show short, thin tubules of ER which occasionally connect with the nuclear envelope (11). When transport from the ER is blocked in a class A sec mutant, the ER tubules and the nuclear envelope expand and the ER winds more extensively through the cytoplasm (11). In contrast to this, sec59 showed fragmented ER tubules of variable width; fragmentation of the nuclear envelope was also observed (Fig. 6A). Mutant cells at 25°C (Fig. 6B), and wild-type cells treated with tunicamycin (not shown) appeared normal. sec53 was not as obviously altered as sec59, although some cells showed rounded vesicles in place of thin ER tubules (not shown).

DISCUSSION

We have identified new yeast secretory mutants that are defective in the processing and transport of exported proteins. The mutants are temperature-sensitive for the production of active secretory forms of invertase and acid phosphatase, but not for the synthesis of active cytoplasmic invertase. These mutants (sec53, sec59) are distinct from our previously reported sec mutants which block export, but not synthesis, of active secretory enzymes (sec1-sec23) (11). A block early in the secretory pathway in sec53 and sec59 is suggested by the incomplete glycosylation of invertase (Figs. 1-3) and by the epistasis of these mutants to sec18, a mutant that blocks transport of active invertase from the ER to the Golgi body (Table III).

Although the mutants do not appear to accumulate membrane, such as is seen in class A sec mutants, the morphology of the ER in sec59 is distinct (Fig. 6). Both the ER and the nuclear membrane are fragmented in contrast to the smooth thin tubules that are seen in sections of wild-type cells. The membrane is not permanently damaged or open, because accumulated invertase precursor is matured and secreted upon return to the permissive temperature (42). Distortion of the ER tubules may result from accumulation of abnormal amounts of partly glycosylated proteins, embedded in or tightly associated with the ER membrane (42).

Invertase cross-reacting material produced in sec53 and sec59 at 37°C ranges from 60-66 kdalton (Fig. 1). In vitro translation studies have shown that invertase is synthesized as a precursor with a leader sequence of ~2 kdalton (23). When invertase mRNA is translated in the presence of dog pancreas microsomes, the leader sequence is cleaved, and 9-10 carbohydrate chains are added to the molecule as it penetrates into the lumen of the ER (23, 42). Each core carbohydrate chain adds ~2 kdalton to the protein portion of invertase which is 60 kdalton. Both sec53 and sec59 accumulate forms of invertase that are >60 kdalton. The diffuse species in sec53 (60-64 kdalton) appears not to contain N-linked carbohydrate: it does not bind to ConA-Sepharose and is not reduced in size by treatment with endo H (Fig. 2 and 3). This form may retain the signal peptide and be modified in some other way to account for a diffuse SDS gel mobility. sec59 accumulates four species (60, 63, 64.5, 66 kdalton): the largest three contain N-linked oligosaccharides, although most of the 63-kdalton species is not glycosylated; the 63-kdalton form may contain the signal sequence. The simplest interpretation of this result is that the sec59 mutation causes invertase penetration to arrest after the addition of 0-3 core oligosaccharides. Current evidence suggests that oligosaccharide transfer from dolichol to polypeptide occurs on the lumenal surface of the ER. Thus, it seems likely that an initial portion of the invertase polypeptide penetrates into the ER lumen in sec59. This possibility is considered further in the accompanying paper (42).

Invertase and CPY differ in the number of protein species that accumulate in sec59. Although CPY has four N-linked oligosaccharides, only one band, with a mobility corresponding to unglycosylated proCPY, accumulates in sec59 (Fig. 5; 39, 11, 10). Indirect evidence suggests that CPY has an amino-terminal 8-kdalton propeptide that is devoid of carbohydrate (40). DNA sequence analysis shows that invertase, in contrast to CPY, has a potential N-glycosylation site at position four of the mature protein sequence (43). Thus, assuming the amino terminus penetrates first across the ER membrane, invertase would present a site for glycosylation shortly after the polypeptide enters the lumen. CPY would not present a glycosylation site until at least 8 kdalton of protein had been translocated.
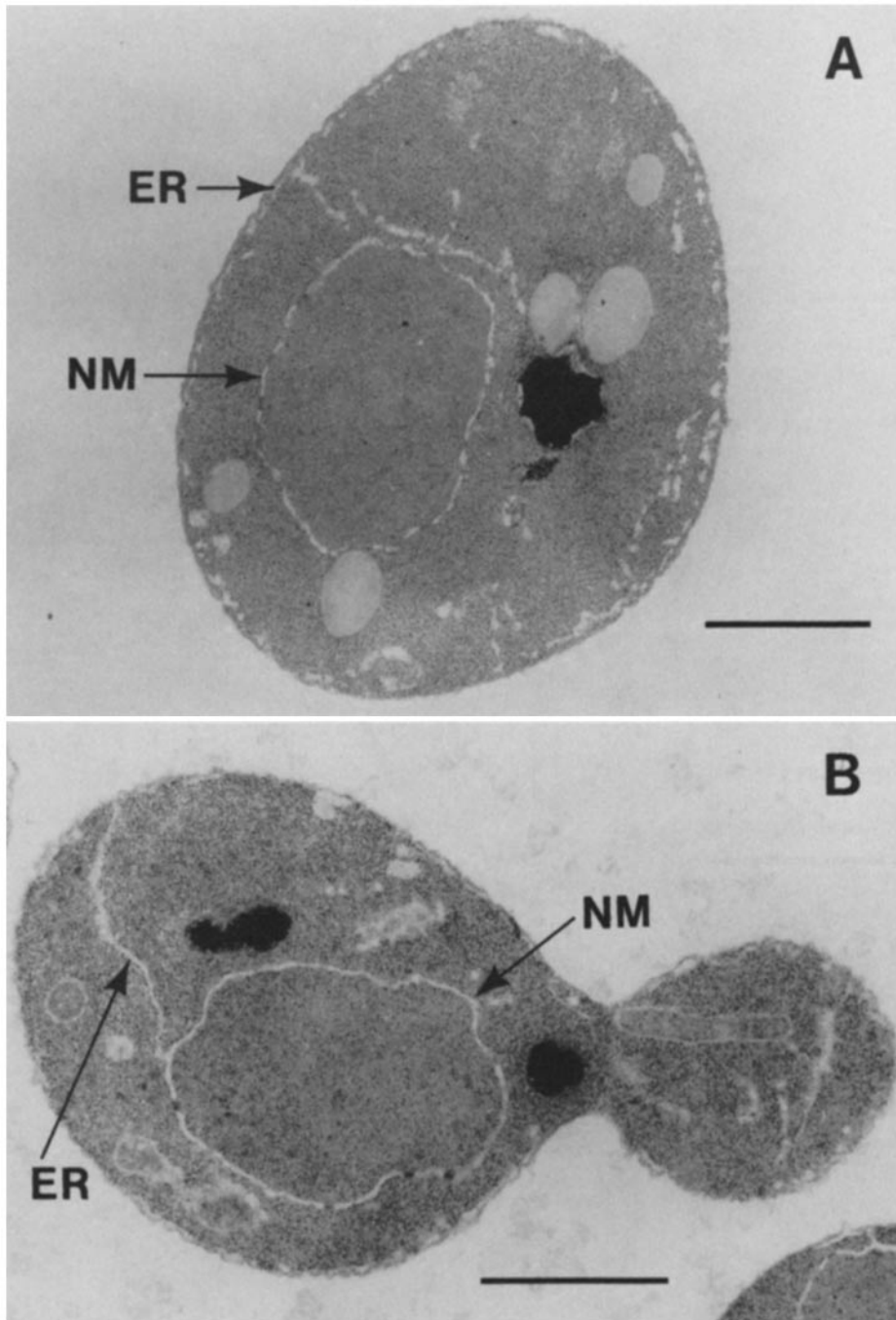
FIGURE 6 Thin section electron micrographs of sec59 (SF409-4D) cells. Cells were grown in YPD medium at (A) 37°C for 3 h and (B) 24°C. ER, endoplasmic reticulum; NM, nuclear membrane. Bar, 0.5 μm.

The behavior of a biosynthetic precursor of the pheromone α-factor is consistent with this pattern. DNA sequence analysis shows that all three potential N-glycosylation sites are in the amino-terminal 40% of an 18-kdalton pro-α-factor (44). As expected from the above hypothesis, sec59 accumulates four immunoreactive species with sizes that correspond to precursors containing 0–3 oligosaccharides (D. Julius, R. Schekman, and J. Thorner, unpublished results).

The glycosylation of proteins can be interrupted by blocking oligosaccharide synthesis directly, such as with tunicamycin, or indirectly by blocking transport of the protein into the ER. Although some of the properties of sec53 and sec59 are mimicked by treatment of wild-type cells with tunicamycin, sulfate permease export is blocked in the mutants but not in drug-treated cells (Table II). Thus, as the mutations and the drug have different effects, they are unlikely to affect the same process. From this we infer that sec53 and sec59 are not directly defective in oligosaccharide synthesis. In support of this concusion, major cell surface proteins that are exported in tunicamycin-treated cells at 25° (27) or 37°C (P. Novick and R. Schekman, unpublished results) are not secreted in sec53 and sec59 at 37°C (Fig. 4).

Inhibition of maturation and transport of secretory, plasma membrane, and vacuolar enzymes in sec53 and sec59 identifies a common step in the translocation of polypeptides across the ER membrane in yeast, as has been shown for analogous proteins in mammalian cells (45, 46). In the accompanying report we provide evidence that this common step occurs

when the transported polypeptide enters the ER membrane.

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