Genes Required for Completion of Import of Proteins into the Endoplasmic Reticulum in Yeast

SUSAN FERRO-NOVICK, WILLIAM HANSEN, IRENE SCHAUER, and RANDY SCHEKMAN

Department of Biochemistry, University of California, Berkeley, California 94720. Dr. Ferro-Novick's present address is Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115.

ABSTRACT Yeast secretory mutants *sec53* and *sec59* define a posttranslational stage in the penetration of glycoprotein precursors into the endoplasmic reticulum (ER). In the previous report we showed that at the restrictive temperature (37°C) these mutants accumulate enzymatically inactive and incompletely glycosylated forms of the secretory enzyme invertase and the vacuolar enzyme carboxypeptidase Y. Cell fractionation experiments reveal that these precursor forms remain firmly bound to the ER membrane. However, upon return to the permissive temperature (24°C), the invertase precursors are glycosylated, become partially active, and are secreted. Thermoreversible conversion does not require protein synthesis, but does require energy. In contrast to the effect of these mutations, inhibition of oligosaccharide synthesis with tunicamycin at 37°C causes irreversible accumulation of unglycosylated invertase. The effect of the drug is exaggerated by high temperature since unglycosylated invertase synthesized in the presence of tunicamycin at 25°C is secreted.

A portion of the invertase polypeptide accumulated at 37°C is preserved when membranes from *sec53* and *sec59* are treated with trypsin. In the presence of Triton X-100 or saponin, the invertase is degraded completely. The protected fragment appears to represent a portion of the invertase polypeptide that is embedded in or firmly associated with the ER membrane. This association may develop early during the synthesis of invertase, so that in the absence of translocation, some of the completed polypeptide chain remains exposed on the cytoplasmic surface of the ER.

In the preceding report we described yeast secretory mutants (*sec53* and *sec59*) that accumulate incompletely processed forms of invertase and carboxypeptidase Y (CPY)[1] (1). The mutants appear to be blocked at some stage after the completion of secretory polypeptide synthesis. One possibility is that *SEC53* and *SEC59* code for components of the signal recognition particle (SRP; 2, 3) or the docking protein (4, 5). Elimination of either SRP or docking protein activity should result in the accumulation of truncated or complete secretory polypeptides in the cytoplasm.

In contrast to the above prediction, we report here that *sec53* and *sec59* accumulate secretory polypeptide precursors that are firmly bound to the endoplasmic reticulum (ER) membrane. The accumulated precursors are authentic intermediates in translocation because they are converted to mature forms and are secreted when mutant cells are returned to the permissive temperature (24°C). Thus, *SEC53* and *SEC59* appear to code for new components of the polypeptide translocation apparatus.

[1] *Abbreviations used in this paper:* CPY, carboxypeptidase Y; DNP, dinitrophenol; ER, endoplasmic reticulum; SRP, signal recognition particle.

MATERIALS AND METHODS

Strains: *sec* mutant strains are described in a previous report (1). Strains containing the *pep4*-3 allele ((6), reduced levels of the major vacuolar proteases) were used in the trypsin experiments. Strains containing the *mnn2* mutation ((7), altered mannan oligosaccharide outer chain) were used for immunoprecipitation of invertase with α-1,6-Man antibody. Growth conditions were the same as described before (1).

Reagents and Buffers: In addition to reagents described in previous reports, pancreatic DNase I (Type I), trypsin, trypsin inhibitor, and saponin were from Sigma Chemical Co. (St. Louis, MO); Iodo-gen was from Pierce

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Chemical Co. (Rockford, IL); dinitrophenol (DNP) was from Mann Research Laboratories (Orangeburg, NY); and Renografin was from E. R. Squibb and Sons (Princeton, NJ). In addition to antibody directed against the secreted form of invertase (1), cytoplasmic (unglycosylated) invertase (8), purified from an invertase plasmid-containing strain grown on minimal medium with 2% glucose, was used to raise antibody. The invertase antibody was purified by absorption and elution from invertase conjugated to Sepharose 4B. The final fraction (0.34 mg/ml) had a titer of 1:8 against cytoplasmic invertase and 1:2 against secreted invertase when tested by the Ouchterlony procedure with 0.25 mg/ml of antigen. Antibody against cytoplasmic invertase was used in the experiments described in Fig. 3, 5, and 6. α-1,6 Antibody (7) was provided by Brent Esmon and Pam Call (University of California, Berkeley).

The following buffers were used in addition to those listed in the previous reports (1, 2): Buffer A is 1.0 M sorbitol, 0.2 M potassium chloride, 5 mM magnesium chloride, 25 mM Tris·HCl (pH 7.5), 1 mM EGTA, 1 mM sodium azide. Buffer B is 0.3 M mannitol, 0.1 M sodium chloride, 5 mM magnesium chloride, 10 mM Tris·HCl (pH 7.5). Sorbitol cushion is 1.7 M sorbitol, 19.7 mM sodium phosphate (pH 7.5). Lysis buffer is 0.2 M mannitol, 0.1 M sodium chloride, 20 mM 2(*N*-morpholino)ethanesulfonic acid (pH 6.5), 10 mM sodium azide.

Cell Fractionation: Mutant and wild-type cells (50 OD_{600} U) were grown and radiolabeled (3 mCi $^{35}SO_4^{2-}$) at 37°C as described previously (1). Parallel cultures of each strain (200 OD_{600} U of cells) were incubated with nonradioactive sulfate and 10 OD_{600} U of each were labeled with ^{125}I under conditions where yeast cell surface proteins, but not cytoplasmic proteins, are iodinated (9, 10). A portion of each iodinated sample (30,000 cpm) was mixed with the corresponding nonradioactive sample, and cells were sedimented from these and the $^{35}SO_4^{2-}$-labeled cultures. Cells were washed with 25 mM Tris·HCl (pH 7.5) and then washed with and resuspended in buffer A at 100 OD_{600} U of cells/ml. After addition of β-mercaptoethanol (40 mM) and lyticase (10^4 U/ml), spheroplasts were formed during a 45 min incubation at 30°C. The spheroplast mixture was layered on 3 vol of 1.2× buffer A and centrifuged for 10 min at 3,000 *g*. Spheroplasts were resuspended in 0.1 ml of buffer B and glass beads (250 mg/100 OD_{600} U of cells) and lysed by agitation on a vortex mixer for 60 s at 4°C. The beads were washed and allowed to settle five times with 0.2-ml aliquots of buffer B and the combined supernatant fractions were adjusted to 1 ml, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mg/ml of pancreatic DNase I. After 30 min at 4°C, EDTA was added to a final concentration of 6 mM and half of each homogenate was layered on a density gradient consisting of the following 2-ml steps: 18, 25, 32, 38, and 43% (wt/vol) sucrose, 43% sucrose + 5% Renografin, 43% sucrose + 11% Renografin, and 43% sucrose + 22% Renografin; all solutions contained 0.4 M sodium chloride, 10 mM 2(*N*-morpholino)ethanesulfonic acid (pH 6.5). Gradients were centrifuged for 3 h at 27,000 rpm in a Beckman SW28.1 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 4°C. Fractions (ten) were collected from the top with the aid of a Searle/Buchler Auto Densi-Flow IIc fractionator (G. D. Searle & Co., Chicago, IL).

Gradient fractions of the iodinated samples were assayed for α-mannosidase, vanadate-sensitive ATPase, NADPH-cytochrome *c* reductase, and ^{125}I cpm. Gradient fractions of the $^{35}SO_4^{2-}$-labeled samples were monitored for newly synthesized protein (^{35}S cpm), and for immunoreactive forms of invertase and CPY; these fractions were also assayed for NADPH-cytochrome *c* reductase. For immunoprecipitation, aliquots of the gradient fractions were adjusted to 1% SDS and heated for 3 min in a boiling water bath. Heated samples (100 μl) were diluted with 1 ml of dilution buffer and treated with 29 μg of anti-invertase IgG (1), or 10 μg of anti-CPY IgG (11). Samples of solubilized immunoprecipitates, proportional to the gradient fraction volumes, were taken for SDS gel electrophoresis. Fluorographs of the gels were scanned and radioactive bands quantified as before (11).

Radiolabeling and Immunoprecipitation of Invertase: Cells were grown as described previously (1). Cell samples (3.8 OD_{600} U) were incubated at 37°C for 30 min, centrifuged in a clinical centrifuge, and resuspended in 1.27 ml of derepression medium (1) containing 460 μCi $^{35}SO_4^{2-}$. After 30 min at 37°C, half the culture was chilled to 0°C. The remaining portion was centrifuged and cells were resuspended in 1.27 ml of minimal medium containing 2% glucose, 1 mM ammonium sulfate, and 100 μg/ml of cycloheximide (chase medium) and incubated at 24°C for 2 h. Both samples were centrifuged and cells were washed with 1.5 ml of 10 mM sodium azide and resuspended in spheroplasting medium (1). After centrifugation, the supernatant fraction that contained secreted proteins was removed and the sedimented spheroplasts were lysed in 1% SDS (1). Invertase was immunoprecipitated from the lysate and the supernatant fractions and analyzed by SDS gel electrophoresis as before (1).

In some experiments tunicamycin (5 μg/ml) was present during both 37°C incubations. In experiments where DNP was used, 3.8 OD_{600} U of radiolabeled cells were washed once with 2 ml of chase medium without glucose and resuspended in 2.54 ml of the same medium. DNP (0.1 mM final concentration) was added to half the culture, and glucose (0.5% final concentration) was added to the other half. Both samples were incubated at 24°C for 2 h.

The addition of mannan outer chain to invertase was evaluated by sequential immune precipitation. Aliquots of invertase immunoprecipitates (30 μl in 1% SDS) were mixed with 1 ml of dilution buffer and 50 μl of IgG Sorb and incubated for 30 min at 0°C to adsorb any remaining invertase antibody. Samples were centrifuged and the supernatant fractions were treated with 50 μg of α,1 → 6-Man IgG. Immunoprecipitation was done as described before (1).

Trypsin-Treatment of Membranes: Cells were grown as described previously (1). Aliquots (20 OD_{600} U) incubated at 37°C for 30 min were sedimented and resuspended in 6.6 ml of derepression medium (1) containing 1–2 mCi of $^{35}SO_4^{2-}$. After 30 min at 37°C, cells were collected by centrifugation, washed once with 2 ml of 10 mM sodium azide, and resuspended in 0.8 ml of spheroplasting medium (with 150 instead of 50 U of lyticase per OD_{600} U of cells). Spheroplasts formed during a 1-h incubation at 30°C were layered on a sorbitol cushion and centrifuged at 5,000 *g* for 10 min. The pellet fraction was resuspended in 0.8 ml of lysis buffer and Dounce-homogenized for five strokes, and the resulting extract was centrifuged at 27,000 *g* for 20 min. Membranes were resuspended in 0.33 ml of lysis buffer with the aid of a Dounce homogenizer; the protein concentration was ~1.2 mg/ml. Samples (25–50 μl) were incubated at 37°C for 30 min and with various concentrations of trypsin in the presence or absence of trypsin inhibitor (0.69 mg/ml), Triton X-100 (0.17%), or saponin (0.17%). Reactions were stopped by the addition of SDS to a final concentration of 1% and the samples were heated for 3 min in a boiling water bath. Solubilized samples were mixed with 1 ml of dilution buffer and centrifuged at 27,000 *g* for 30 min. Supernatant solutions were mixed with 84–168 μl of affinity-purified cytoplasmic invertase antibody, and immunoprecipitation was performed as before (1). Solubilized immunoprecipitates were analyzed by electrophoresis on 17% polyacrylamide SDS gels as described before (1). Molecular mass standards for the 17% polyacrylamide SDS gels were: lysozyme (14.6 kdalton), cytochrome *c* (12.5 kdalton), aprotinin (6.5 kdalton), and standards listed previously (1).

Enzyme Assays and Other Procedures: External invertase was assayed as previously described (8). α-Mannosidase was assayed as described by Opheim (12), except the reaction was terminated by the addition of 0.1 ml of 36% zinc sulfate, 0.1 ml of 7.5% barium hydroxide, and 0.05 ml of 4 N sodium hydroxide, followed by centrifugation in a Fisher microcentrifuge. Vanadate-sensitive Mg^{2+} ATPase was assayed as described by Serrano (13), except the ATP concentration was 6 mM, the Mg^{2+} concentration was 12 mM, and the reaction was terminated with acid molybdate containing 5% (wt/vol) SDS. NADPH-cytochrome *c* reductase was assayed as described by Kubota et al. (14). ^{125}I was quantified with a Searle 1185 automatic gamma system (G. D. Searle & Co.).

RESULTS

Intracellular Localization of Accumulated Precursors

Cell fractionation was used to determine the intracellular location of immunoreactive forms of invertase and CPY that accumulated at 37°C in *sec53* and *sec59*. Cells grown in minimal medium + 5% glucose were transferred to minimal medium + 0.1% glucose (to derepress invertase synthesis) for 30 min at 37°C and radiolabeled with $^{35}SO_4^{2-}$ for 30 min at the same temperature. Spheroplasts were prepared and lysed by osmotic shock and agitation with glass beads, and the entire lysate was centrifuged on a step gradient. Lysates of surface-iodinated cells were centrifuged in parallel and used to monitor sedimentation of marker enzymes. Vanadate-sensitive Mg^{2+} ATPase and ^{125}I cpm were used as plasma membrane and cell surface markers, respectively (10, 13); NADPH-cytochrome *c* reductase was used as an ER marker (14); α-mannosidase was used as a vacuolar membrane marker (12); ^{35}S cpm was used to mark newly synthesized protein. Gradient fractions derived from $^{35}SO_4^{2-}$-labeled samples were treated with SDS and mixed with anti-invertase and anti-CPY IgG. Immunoprecipitates were solubilized and analyzed by SDS gel electrophoresis. Table I shows the gradient profiles of marker enzymes, ^{125}I-label, ^{35}S total, and ^{35}S-labeled

TABLE I

Fractionation of Membranes from sec53 and sec59

	Gradient fractions									
	1 (Top)	2	3	4	5	6	7	8	9	10 (Bottom)
	*% of all fractions**									
	sec53									
Marker‡										
^{35}S cpm	31	41	6	3	5	5	8	2	0.3	1
α-Mannosidase	3	71	16	4	2	1	2	1	1	1
NADPH-cytochrome c reductase	6	9	15	11	19	14	22	4	1	0
Invertase§	1	3	2	4	21	27	37	5	1	1
CPY	1	6	2	5	22	23	36	4	1	1
Vanadate-sensitive Mg^{2+}-ATPase	0	3	2	6	4	7	66	10	1	2
^{125}I cpm	9	10	7	4	5	5	13	20	21	7
	sec59									
^{35}S cpm	24	50	5	4	3	4	8	1	0.4	0.4
α-Mannosidase	4	73	12	3	3	1	2	1	0	0
NADPH-cytochrome c reductase	3	12	8	8	15	13	29	9	1	2
Invertase	1	4	2	6	18	33	35	2	0	0
CPY	0	3	10	3	13	17	49	4	1	0
Vanadate-sensitive Mg^{2+}-ATPase	0	0	3	2	11	2	49	31	2	1
^{125}I cpm	5	15	3	2	2	2	10	23	29	8

* Gradient fractions were assayed as described in Materials and Methods. Numbers represent the percentage recovered relative to the entire gradient; in all cases the recovery was >60% with respect to the starting fraction.

‡ Samples applied to the gradient contained the following amounts of each marker: for *sec53* (SF649-2A/pRB58), ^{35}S, 2.7×10^8 cpm; α-mannosidase, 15.3 U; NADPH-cytochrome c reductase, 0.17 U; vanadate-sensitive Mg^{2+} ATPase, 0.37 U; ^{125}I, 8×10^3 cpm. The corresponding amounts in the *sec59* (SF629-3C/pRB58) sample were 1.9×10^8 cpm, 2.2 U, 0.17 U, 0.38 U, 9.1×10^3 cpm.

§ Invertase and CPY relative amounts were quantified from densitometric scans of fluorographs. For *sec59*, the four invertase species were summed.

invertase and CPY, derived from *sec53* and *sec59* lysates. Most of the newly synthesized protein was in fractions *1* and *2*; α-mannosidase was mainly in fractions *2* and *3*; NADPH-cytochrome *c* reductase was enriched in fractions *5–7*; vanadate-sensitive Mg^{2+} ATPase was in fractions *7* and *8*; ^{125}I surface label was in fractions *7–10*. Invertase and proCPY co-sedimented in both samples in fractions *5–7*; this distribution coincided with the ER marker enzyme that sedimented to the same position in gradients of the ^{125}I- and ^{35}S-labeled samples. Furthermore, the four invertase species accumulated in *sec59* co-sedimented in constant proportion. Membranes prepared from wild-type cells treated with tunicamycin at 37°C also showed immunoreactive invertase and CPY enriched in the ER fractions (not shown). Thus, by this criterion, accumulated precursor forms of invertase and CPY remain associated with ER membranes.

In the previous report we showed that *sec53* accumulates a 60–64-kdalton form of invertase, while *sec59* produces four discrete species of 60, 63, 64.5, and 66 kdalton (1). Since all these forms appeared to remain ER-associated during sucrose gradient sedimentation, we tested the nature of the membrane interaction with several solubilization techniques. Cells were labeled at 37°C and lysed directly with glass beads and SDS, or first converted to spheroplasts and then lysed with 1% Triton X-100. The Triton lysates were centrifuged and the pellet fraction was solubilized in 1% SDS. Fig. 1 shows an SDS gel fluorograph of radiolabeled invertase that was immunoprecipitated from solubilized fractions. Only a 60-kdalton species labeled in *sec53* was solubilized by Triton X-100; this probably corresponded to the soluble cytoplasmic form of invertase (15). The 60–64-kdalton form required SDS for solubilization. SDS was also required for efficient solubilization of the four species labeled in *sec59*; again only a 60-kdalton species was released from spheroplasts by Triton X-100. Wild-type cells treated with tunicamycin produce mainly a 60-kdalton form of invertase, although a small amount of a 63-kdalton species is seen (1). Some of the 60-kdalton form was solubilized by Triton, but most required SDS; all of the 63-kdalton form required SDS for solubilization. Thus, the results of membrane fractionation and detergent solubilization taken together suggest that secretory precursor forms of invertase produced in the mutants and in wild-type cells treated with tunicamycin were stuck tightly to or embedded in the ER membrane.

Secretory polypeptides appear to become lodged in the ER at somewhat different stages of membrane penetration in *sec53* and *sec59*. If these *sec* gene products act at saturable sites that become jammed at 37°C, prolonged incubation might lead to accumulation of secretory polypeptides in the cytoplasm. This possibility was tested with mutant cells incubated for 30 or 90 min at 37°C prior to derepression of invertase synthesis. Cells were labeled with $^{35}SO_4^{2-}$ for 30 min at 37°C during invertase derepression, spheroplasts were prepared and lysed in sodium phosphate buffer, and a membrane fraction was collected by centrifugation. Invertase was immunoprecipitated from the soluble fraction and from an SDS-solubilized membrane fraction, and solubilized immunoprecipitates were evaluated by SDS gel electrophoresis. The distribution between soluble and membrane forms of invertase did not change during extended incubation at 37°C. A change was seen, however, in the pattern of invertase forms in the *sec59* membrane fraction: after 2 h at 37°C, the 66- and 64.5-

kdalton glycosylated species were less prominent (data not shown). A similar observation was made for α-factor; a pronounced reduction in the number of immunoreactive species that accumulate in *sec59* were seen (D. Julius, R. Schekman and J. Thorner, unpublished results).

Thermoreversible Block in sec53 and sec59

If the invertase precursor forms are true intermediates in the process of protein translocation into the ER, then completion of the import process should be achieved when the *sec53* and *sec59* functions are restored. Mutant cells were labeled with $^{35}SO_4^{2-}$ for 30 min at 37°C under conditions that derepress the synthesis of invertase. A portion of each culture was returned to 24°C in medium containing excess SO_4^{2-} and cycloheximide. After the recovery incubation at 24°C, cells were converted to spheroplasts and centrifuged. The spheroplasts were lysed in SDS. Invertase in the spheroplast supernatant fraction (secreted proteins) and in the spheroplast lysate (accumulated precursors) was evaluated by immunoprecipitation and SDS gel electrophoresis. Fig. 2 shows that the precursors accumulated at 37°C and that only a small amount was recovered in the spheroblast supernatant fraction; this small amount could have been due to secretion or lysis during spheroplast formation (*sec53*, lanes *1* and *3; sec59*, lanes *5* and *7*). After 2 h at 24°C the pattern was quite different; most of the invertase was secreted and was in a heterogeneous and low electrophoretic mobility form (*sec53*, lane *4; sec59*, lane *8*). The material retained in the spheroplasts, after incubation at 24°C, was also heterogeneous but was of higher average electrophoretic mobility (*sec53*, lane *2; sec59*, lane *6*).

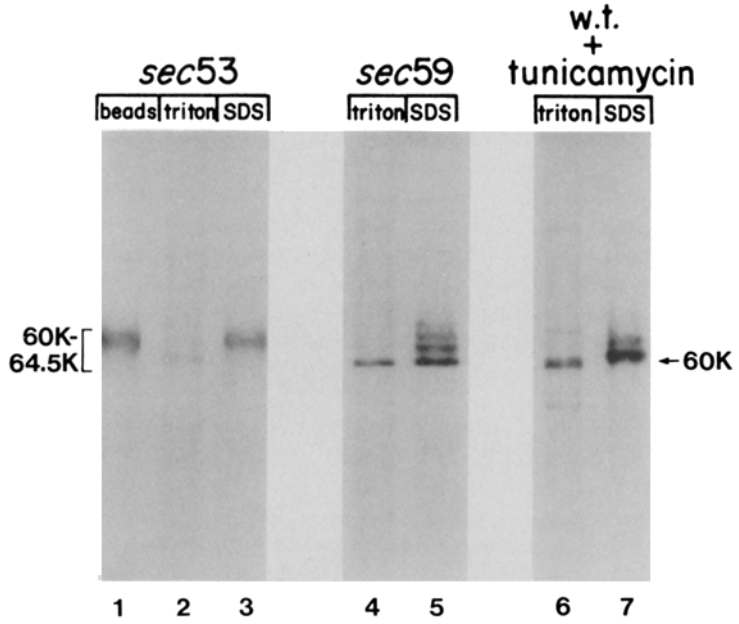

FIGURE 1 Solubilization of accumulated invertase cross-reacting material. Cells were labeled at 37°C and either lysed with glass beads and SDS, or first converted to spheroplasts and then sequentially extracted with Triton X-100 and SDS. Invertase was immunoprecipitated from solubilized fractions and analyzed by SDS polyacrylamide (10%) gel electrophoresis. A fluorograph of the gel is shown. (lane *1*) *sec53* (SF649-2A/pRB58) lysate with glass beads; (lane *2*) same, Triton-soluble fraction from spheroplasts; (lane *3*) same, SDS-soluble fraction from spheroplasts; (lane *4*) *sec59* (SF629-3C/pRB58) Triton-soluble; (lane *5*) same, SDS-soluble; (lane *6*) wild type (SF610-7B;pRB58) treated with tunicamycin, Triton-soluble; (lane *7*) same, SDS-soluble.

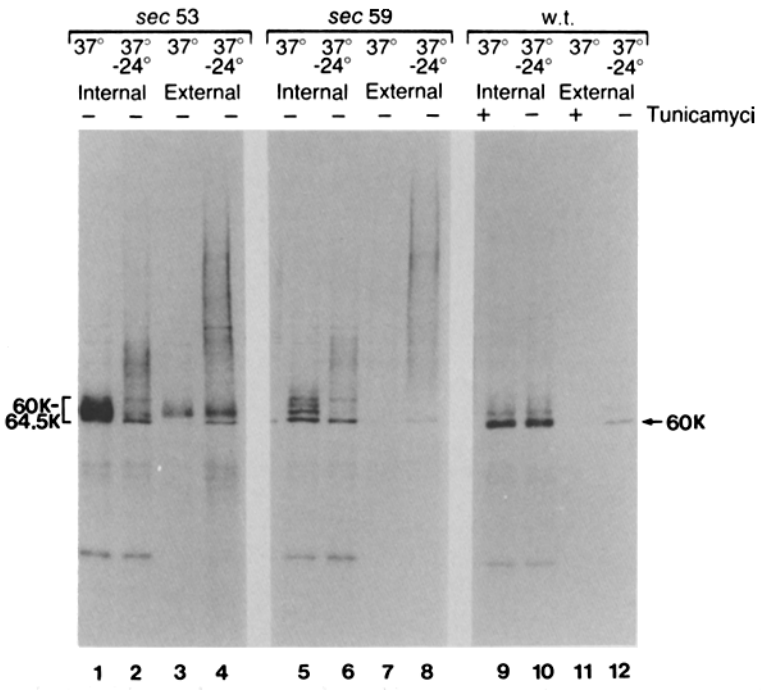

FIGURE 2 Thermoreversible accumulation of immunoreactive invertase in *sec53* and *sec59*. Cells were labeled with $^{35}SO_4^{2-}$ at 37°C and half of each sample was shifted to 24°C for 2 h in the presence of cycloheximide (100 μg/ml). Spheroplasts were prepared, sedimented, and lysed with SDS. Invertase was immunoprecipitated from the lysate and spheroplast supernatant fractions. Solubilized immunoprecipitates were analyzed by SDS gel electrophoresis (1); a fluorograph of the gel is shown. (lane *1*) *sec53* (SF649-2A/pRB58) 37°C, spheroplast lysate; (lane *2*) *sec53* 37°C shifted to 24°C, spheroplast lysate; (lane *3*) *sec53* 37°C, spheroplast supernatant; (lane *4*) *sec53* 37°C shifted to 24°C, spheroplast supernatant; (lane *5*) *sec59* (SF629-3C/pRB58) 37°C, spheroplast lysate; (lane *6*) *sec59* 37°C shifted to 24°C, spheroplast lysate; (lane *7*) *sec59* 37°C, spheroplast supernatant; (lane *8*) *sec59* 37°C shifted to 24°C, spheroplast supernatant; (lane *9*) wild type (SF610-7B/pRB58) 37°C (with tunicamycin), spheroplast lysate; (lane *10*) wild type 37°C (with tunicamycin) shifted to 24°C (without drug), spheroplast lysate; (lane *11*) wild type 37°C (with tunicamycin), spheroplast supernatant; (lane *12*) wild type 37°C (with tunicamycin) shifted to 24°C (without drug), spheroplast supernatant.

TABLE II

Invertase Activity after the Recovery Incubation

Strain	Genotype	Derepressed at 37°C	Derepressed at 37°C and shifted to 24°C
		*U/mg dry wt**	*ΔU/mg dry wt**
SF649-2A/pRB58	*sec53*	−0.01‡	0.26
SF629-3C/pRB58	*sec59*	−0.09	0.08
SF610-7B/pRB58	Wild type	1.04	1.18

* Mutant and wild-type strains carrying the *SUC2* plasmid (35) were grown to early exponential phase in minimal medium supplemented with sulfate, histidine, lysine, and 5% glucose (1). An aliquot of cells (1.9 OD_{600} U) was chilled, sedimented, and resuspended in 1 ml of 10 mM sodium azide; these cells were used to measure the starting invertase activity. Another aliquot (3.8 OD_{600} U) was incubated for 30 min at 37°C; cells were then sedimented in a clinical centrifuge and resuspended in 1.27 ml of minimal medium supplemented with sulfate, histidine, lysine, and 0.1% glucose (to derepress invertase; [1]), and incubated at 37°C for 30 min longer. At the end of the 37°C incubation half of the sample was added to a tube containing 1 mg of glucose and 0.1 mg of cycloheximide and the mixture was incubated at 24°C for 2 h; cells in the remaining portion of the culture were chilled, sedimented, and resuspended in 1 ml of 10 mM sodium azide. At the end of the incubation the remaining samples were also chilled and resuspended in sodium azide. Whole cells were assayed for invertase activity. The invertase activity of the starting sample was subtracted from the final value.

‡ The invertase activity of the starting sample (repressed level) was subtracted from the final value. Negative numbers were obtained because the repressed level of the enzyme was diluted by cell growth.

The range in electrophoretic mobility of the forms secreted during the recovery at 24°C was greater than that seen for normal secreted invertase (1, 16).

The invertase that accumulates at 37°C in *sec53* and *sec59* is inactive (1). After a return to 24°C, in the presence of cycloheximide, both *sec53* and *sec59* secreted invertase that had some enzymatic activity (Table II). Assays of whole cells, which detect only secreted activity, showed that *sec53* cells secreted 22%, and *sec59* cells secreted 7%, of the invertase activity secreted by the wild-type strain. These values may be higher if the lower rate of protein synthesis in the mutant strains is taken into account (50 and 68% relative to wild-type for *sec53* and *sec59*, respectively [1]).

Invertase secreted during the recovery incubation at 24°C displayed a heterogeneous electrophoretic mobility. Heterogeneity in invertase secreted by wild-type strains is attributable to the outer chain oligosaccharde (16). This structure is added to core oligosaccharide-containing glycoproteins in the Golgi body (17). An independent test for the presence of the outer chain relies on a unique immunochemical determinant that is detected in certain oligosaccharde processing mutants. In one mutant, *mnn2*, the α,1→6 backbone of the outer chain, which is extensively substituted in normal strains, is exposed. In *mnn2* cells, only glycoproteins that have been transported through the Golgi body can be precipitated by α,1→6-Man antibody (17).

Haploid double-mutant strains containing the *mnn2* and *sec53* and *sec59* mutations were labeled with $^{35}SO_4^{2-}$ at 37°C for 30 min; portions of each culture were then transferred to 24°C for 2 h in the presence of cycloheximide. Cells were converted to spheroplasts and centrifuged, and invertase was immunoprecipitated from spheroplast supernatant fractions, and from spheroplast lysates. Solubilized and outer chain-containing invertase was reprecipitated with a,1→6-Man antibody. The results showed that invertase accumulated in the mutants of 37°C did not contain outer chain, but that invertase secreted during the recovery incubation at 24°C did (data not shown).

Wild-type cells treated with tunicamycin at 37°C also accumulated immunoreactive invertase (Fig. 2, lanes *9* and *11*). This material is insoluble in Triton X-100, and remains associated with the ER membrane. When tunicamycin-treated cells were transferred to 24°C in drug-free medium, no further glycosylation, and little secretion of the accumulated invertase, was detected (Fig. 2, lanes *10* and *12*).

Vesicular stomatitis virus G protein, produced in the presence of tunicamycin, is exported to the plasma membrane of infected tissue culture cells at 25°C, but not at 37°C (18). This effect is virus strain-dependent, thus reflecting some structural feature of the G protein, and not a requirement for glycosylation in the export process. The effect of temperature on secretion of unglycosylated invertase was examined with wild-type cells radiolabeled at 25°C in the presence of tunicamycin. After a 15-min pulse with $^{35}SO_4^{2-}$ at 25°C, the culture was supplemented with excess SO_4^{2-}, glucose, and cycloheximide and the incubation was continued. At the times indicated (Fig. 3) samples were removed and cells were converted to spheroplasts and centrifuged. Sedimented spheroplasts were lysed with SDS, and the distribution of invertase in the spheroplast supernatant fractions and the spheroplast lysates was examined by immunoprecipitation. At 25°C, about one-half of the unglycosylated invertase was secreted within the first 20 min of chase, quite a contrast from the irreversible accumulation of unglycosylated invertase at 37°C (Fig. 2). Thus, as seen with the vesicular stomatitis virus G protein, glycosylation is not required for invertase transport.

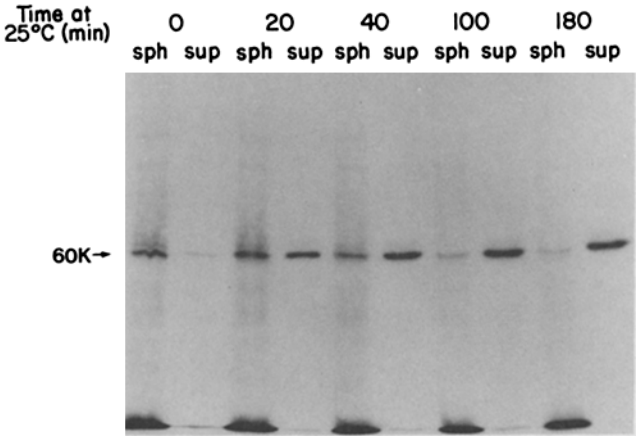

FIGURE 3 Secretion of unglycosylated invertase at 25°C. Wild-type cells (X2180-1A) grown in minimal medium (1) at 25°C were transferred to invertase derepressing medium (1) (at 2 OD_{600} U of cells/ml) containing 10 μg/ml tunicamycin. After 30 min at 25°C, $^{35}SO_4^{2-}$ (250 $\mu C_i/A_{600}$ cells) was added. A chase was initiated after 15 min of labeling by the addition of glucose to 0.5%, cycloheximide to 0.1 mg/ml, and $(NH_4)_2SO_4$ to 1 mM. Samples were taken at the indicated chase times. Cells were sedimented, converted to spheroplasts, and centrifuged. The spheroplast pellet fraction (*sph*) was lysed in SDS. Lysates and spheroplast supernatant fractions (*sup*) were treated with cytoplasmic invertase antibody. Solubilized immunoprecipitates were analyzed by SDS gel electrophoresis; a fluorograph of the gel is shown. Total ^{35}S-protein in the supernatant fraction declined from 0 to 20 min and then remained constant, indicating that spheroplast lysis did not account for recovery of invertase in the supernatant fraction during the chase.

Reversible Maturation Is Energy-dependent

We have shown previously that energy is required for reversible secretion of active invertase accumulated in the class A *sec* mutants (19). In these studies, ATP levels were lowered 10-fold by incubating cells in medium containing 0.1 mM DNP and no glucose. Similar conditions were used to examine the energy dependence of precursor maturation in *sec53* and *sec59*. If translocation of the invertase precursor and addition of core oligosaccharides did not require energy, soluble invertase containing 9–10 core units would accumulate in the presence of DNP.

Mutant cells were radiolabeled with $^{35}SO_4^{2-}$ in derepression medium (1) for 30 min at 37°C. A portion of each culture was chilled, and the remaining cells were washed and resuspended in chase medium containing cycloheximide and no glucose. DNP (0.1 mM final concentration) was added to half the culture and glucose (0.5% final concentration) was added to the remaining portion. Both samples were incubated at 24°C for 2 h. Cells were converted to spheroplasts and the secreted and accumulated forms of invertase were examined by immunoprecipitation and SDS gel electrophoresis. Fig. 4 shows that DNP blocked both glycosylation and secretion of the precursor accumulated in *sec53*; similar results were obtained with *sec59* (not shown). The invertase precursor that remained within the DNP-treated cells had the same electrophoretic mobility as the initial precursor (lanes *1* and *3*), and remained insoluble in Triton X-100 (not shown). Thus, energy is required either directly or indirectly to complete translocation of the invertase precursor into the lumen of the ER.

Membrane-protected Fragments of Invertase

Results presented thus far suggest that invertase accumulated in *sec53* and *sec59* remains embedded in the ER membrane. Furthermore, of the 9–10 core oligosaccharide units that are normally attached to invertase during penetration into the ER, only 0–3 units are added in the mutants at 37°C. Either a significant fraction of the invertase protein remains on the cytoplasmic surface of the ER membrane, or protein penetration is uncoupled from glycosylation. To distinguish between these possibilities, membranes from mutant cells were treated with trypsin and protected fragments were evaluated by immunoprecipitation with antibody directed against cytoplasmic invertase.

Mutant cells were radiolabeled in derepression medium at 37°C and cells were converted to spheroplasts. Sedimented spheroplasts were lysed by osmotic shock in a buffer (2(*N*-morpholino)ethanesulfonic acid, pH 6.5) that contained 0.2 M mannitol and 0.1 M sodium chloride, and membranes were concentrated by centrifugation. Resuspended membranes (~1.2 mg protein/ml) were treated with trypsin in the presence or absence of 0.17% Triton X-100 or 0.17% saponin. After treatment, samples were solubilized with SDS, and immunoprecipitated invertase was analyzed by electrophoresis on 17% polyacrylamide SDS gels. At this concentration of polyacrylamide, the invertase precursor migrated only part way into the gel (Fig. 5, lane *1*). At high concentrations of trypsin (350 μg/ml), several low molecular mass fragments of invertase were detected (Fig. 5*A*, lanes *4* and *6*). For *sec53*, fragments of 10.5, 9.4, and 8.4 kdalton were seen; the 10.5 and 8.4-kdalton fragments predominated in membranes from *sec59*. Proteolysis was enhanced considerably by the addition of Triton X-100 or saponin, even though Triton X-100 did not solubilize the invertase precursor (Fig. 5*A*, lanes *5* and *7*; Fig. 5*B*, lanes *2* and *3*). Degradation of the largest fragment was most pronounced in the presence of detergent. Control experiments showed that the fragments were not produced when membranes were incubated alone, with Triton X-100 only, or with trypsin and trypsin inhibitor (Fig. 5*A*, lanes *1–3*). Membranes prepared from wild-type cells treated with

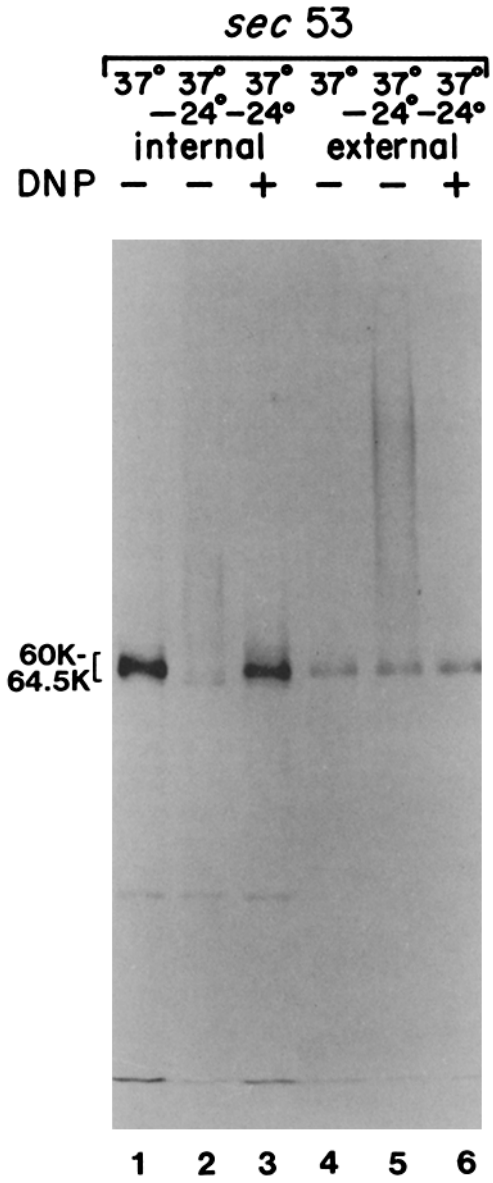

FIGURE 4 Reversible secretion of invertase is blocked by DNP. A culture of *sec53* (SF649-2A/pRB58) was labeled with $^{35}SO_4^{2-}$ for 30 min at 37°C. An aliquot was chilled and the remaining culture was split and resuspended in chase medium (with 0.5% instead of 2% glucose) or in chase medium with DNP (0.1 mM) and without glucose and incubated for 2 h at 24°C. Cells were converted to spheroplasts and lysed in SDS. The lysates and spheroplast supernatant fractions were treated with invertase antibody. Solubilized immunoprecipitates were analyzed by SDS gel electrophoresis; a fluorograph of the gel is shown. (lane *1*) 37°C without DNP, spheroplast lysate; (lane *2*) 37°C shifted to 24°C without DNP, spheroplast lysate; (lane *3*) 37°C shifted to 24°C with DNP, spheroplast lysate; (lane *4*) 37°C without DNP, spheroplast supernatant; (lane *5*) 37°C shifted to 24°C without DNP, spheroplast supernatant; (lane *6*) 37°C shifted to 24°C with DNP, spheroplast supernatant.

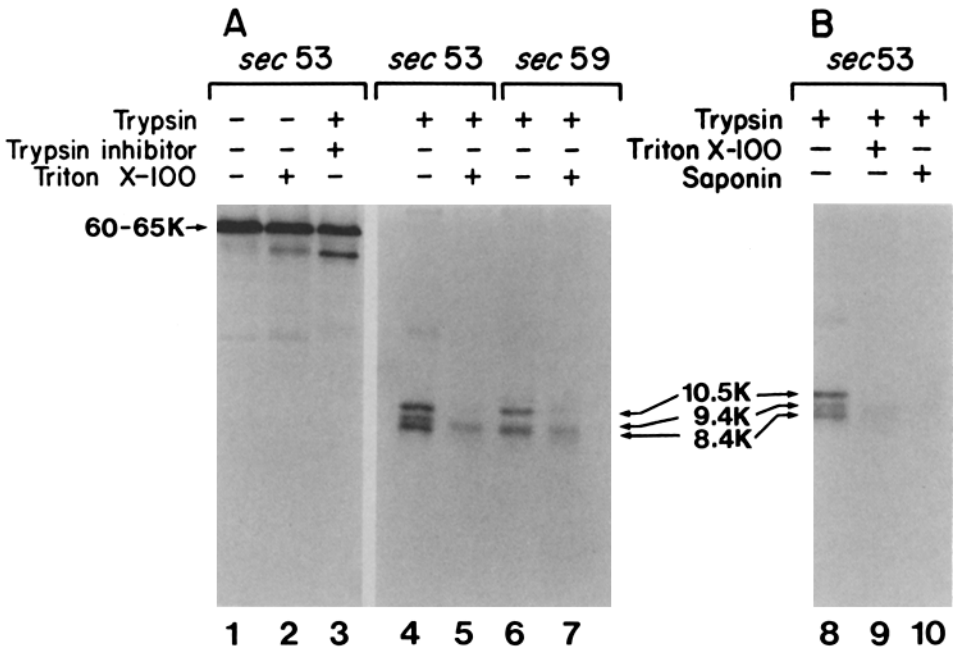

FIGURE 5 A portion of the membrane-bound invertase polypeptide is resistant to proteolysis. Membranes were prepared by osmotic lysis of spheroplasts from mutant cells (*sec53*, SF649-7B/pRB58; *sec59*, SF629-2D/pRB58; wild type, SF610-2D/pRB58) labeled at 37°C with $^{35}SO_4^{2-}$. Membranes (~1.2 mg/ml) were treated without or with trypsin (0.35 mg/ml) with the indicated additions: (*A*) trypsin inhibitor (0.69 mg/ml) or Triton X-100 (0.17%); (*B*) Triton X-100 (0.17%) or saponin (0.17%). All incubations were for 30 min at 37°C. Incubated samples were heated after the addition of SDS, diluted, and treated with cytoplasmic invertase antibody. Solubilized immunoprecipitates were analyzed by electrophoresis on a 17% polyacrylamide SDS gel. Fluorographs of the gels are shown. Strains used in this experiment contained a mutation (*pep4-3*) that lowers the level of the major yeast protease activities (6).

tunicamycin at 37°C produced the same invertase fragments as seen with *sec59* (not shown).

Antibody elicited with the secreted form of invertase precipitated only the 10.5-kdalton fragment. Immunoprecipitation of the radioactive fragment was competed effectively with nonradioactive glycosylated invertase (not shown). With this antibody we also observed enhanced degradation of the 10.5-kdalton fragment when membranes were treated with trypsin in the presence of Triton X-100. Furthermore, in the absence of detergent, the 10.5-kdalton fragment sedimented along with membranes (not shown).

In contrast to the membrane-bound precursors, soluble cytoplasmic invertase did not generate the 10.5-kdalton fragment when treated with high concentrations of trypsin. The fragments produced in this reaction were not altered by degradation in the presence of Triton X-100.

A number of higher molecular mass invertase fragments were produced when membranes from the mutants and from tunicamycin-treated wild-type cells were incubated with a lower concentration of trypsin (18.4 μg/ml). Fig. 6 shows that at this concentration of trypsin, no intact precursor remained and the 10.5-kdalton fragment was already a prominent species. At trypsin concentrations <18.4 μg/ml, intact precursor remained even if Triton X-100 was present during the treatment (not shown). Thus, from the minimum concentration of trypsin needed to clip all the precursor, to the maximum concentration tested (a nearly 20-fold range of trypsin), the 10.5-dalton fragment remained membrane-protected.

No marker for the lumenal surface of the yeast ER has been described. Thus, we were unable to devise an independent test to demonstrate that ER membrane vesicles isolated from *sec53* and *sec59* were sealed with the cytoplasmic surface facing out. It was possible, however, to demonstrate that the lysis procedure preserved intact ER vesicles from a class A *sec* mutant. *sec18* accumulates active invertase in the ER lumen (20). Lysis and isolation of membranes from *sec18* cells, using the same conditions described for membranes from *sec53* and *sec59*, allowed recovery of 85% of the derepressed invertase activity in a sedimentable fraction. The invertase was released in a soluble form by mild treatments such as hypnotic lysis in water, or low concentrations of nonionic detergents (I. Schauer, unpublished results). Isolated vesicles were only partly permeable to sucrose because invertase activity was stimulated ~50% by Triton X-100, whereas cytoplasmic invertase activity was not stimulated by detergent. This latency was reduced only 5% by the low concentration of trypsin used to demonstrate accessibility of the invertase precursor (Fig. 6). ER vesicles were still latent after treatment with high concentrations of trypsin (Fig. 5). A similar lysis procedure generates apparently intact ER vesicles from wild-type cells (21).

DISCUSSION

The structure and ER-membrane association of invertase accumulated in *sec53* and *sec59* suggests that a rather late step in the membrane translocation reaction is blocked. Early steps in the translocation of mammalian secretory proteins appear to involve interaction of the signal sequence on a nascent polypeptide with a ribonucleoprotein complex, the SRP, that guides the ribosome complex to the ER membrane (2, 3). A docking protein on the cytoplasmic surface of the ER discharges the SRP allowing completion of secretory protein translation (4, 5). Elimination of SRP in reconstituted reactions leads to the formation of soluble, unsequestered secretory precursor polypeptides (22); elimination of docking protein results in the synthesis of incomplete secretory polypeptides (4). Thus, assuming this mechanism operates in yeast, mutations that affect SRP or docking protein would probably lead to accumulation of soluble forms of invertase. From this we infer that *SEC53* and *SEC59* do not code for components of SRP or docking protein.

Escherichia coli mutants that lead to accumulation of cytoplasmic precursor forms of secreted proteins have been

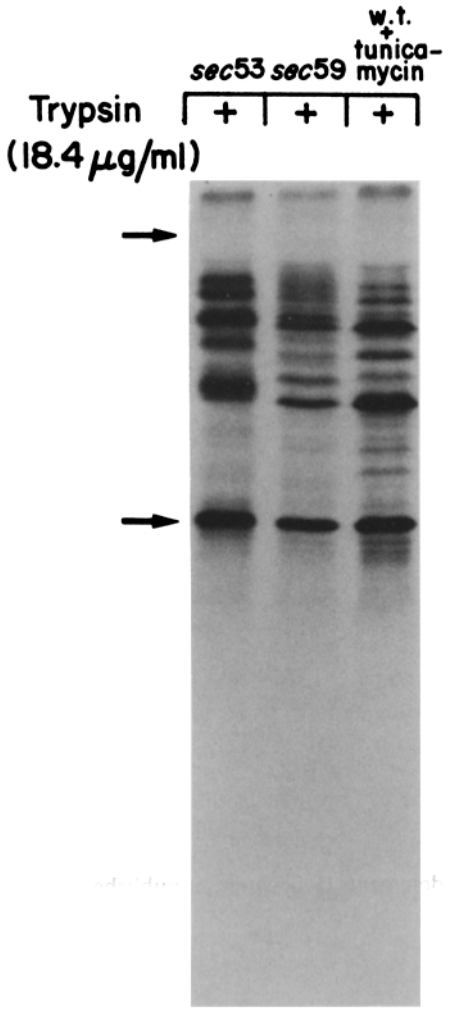

FIGURE 6 All of the precursor invertase is nicked by low levels of trypsin. Radiolabeled membranes were prepared from mutant cells and from wild-type cells treated with tunicamycin at 37°C, and incubated with trypsin at 18.4 μg/ml for 30 min at 37°C. Immunoprecipitation was performed with antibody directed against the cytoplasmic form of invertase. Solubilized immunoprecipitates were analyzed by electrophoresis on a 17% polyacrylamide SDS gel. A fluorograph of the gel is shown. The arrow at the top of the gel shows where intact precursor migrates; the arrow at the bottom of the gel shows where the 10.5-kdalton fragment migrates.

described by Beckwith and co-workers (23, 24). They took advantage of the observation that a hybrid protein containing the maltose binding protein at the N-terminus and β-galactosidase at the C-terminus abortively enters the cytoplasmic membrane of *E. coli* and restricts the formation of enzymatically active β-galactosidase. Mutations in two cellular genes, *secA* and *secB*, allow the hybrid protein to express β-galactosidase activity by blocking transfer of the hybrid protein into the membrane. Certain *secA* mutants are conditionally lethal and accumulate soluble precursors of other exported proteins. The selection for active β-galactosidase may not have detected mutants that block late steps in membrane translocation.

Enzymatically inactive precursors of invertase that accumulate in *sec53* and *sec59* at 37°C are glycosylated, become partially active, and are secreted when cells are returned to 24°C (Fig. 2, Table II). Maturation and secretion of the precursors do not depend on protein synthesis, thus the mutants define a stage in polypeptide translocation that follows completion of the polypeptide chain.

Invertase precursor maturation requires energy (Fig. 4). Inhibition of processing by DNP could be due to a block in penetration across the ER membrane, or inhibition of the synthesis or transfer of the core oligosaccharides. Energy in the form of membrane potential is required for the assembly and processing of a variety of membrane and periplasmic proteins in bacteria (25–27) and for localization of mitochondrial proteins in yeast (28–30). No energy requirement has as yet been reported for translocation of proteins into the ER.

Invertase glycosylation accompanies secretion of the accumulated precursors. Although glycosylation is not required for secretion of invertase at 25°C, inhibition of oligosaccharide synthesis at 37°C causes irreversible accumulation of inactive enzyme (Figs. 2 and 3 and reference 1). Oligosaccharides may be important at the high temperature in facilitating proper folding of the polypeptide during membrane penetration. This is not a general requirement for the formation of active invertase because the cytoplasmic enzyme is produced normally at 37°C (1).

Evaluation of the role played by the *SEC53* and *SEC59* gene products depends critically on the orientation of invertase precursors accumulated in the mutants. If the *SEC53* and *SEC59* gene products are required early in the penetration of invertase across the ER membrane, most of the precursor polypeptide will remain on the cytoplasmic surface of the ER. If, on the other hand, protein penetration is blocked at a late stage, and core glycosylation is reversibly uncoupled from import, the mutants will accumulate precursors sequestered within the ER lumen. These possibilities were addressed by treating membranes with trypsin followed by antibody precipitation of protected, immunoreactive fragments. The results show that most of the invertase polypeptide is susceptible to trypsin, but a portion is protected unless low concentrations of detergent are present during proteolysis (Figs. 5 and 6).

Three possible interpretations of the trypsin results are presented in Fig. 7; the arrows indicate the positions of trypsin cleavage that generate protected immunoreactive fragments. The models in Fig. 7, *A* and *B* assume that the N-terminus penetrates first and is protected by the membrane. Perhaps coincidentally, the sequence of the invertase gene predicts a trypsin cleavage site that would generate an 8.4-kdalton fragment from the N-terminus of the mature protein, and a 10.5-kdalton fragment from the N-terminus of the precursor polypeptide (31). That fragments of these sizes are seen in membranes from *sec53* and *sec59* suggests that many invertase molecules are arrested at the same stage in translocation in both mutants.

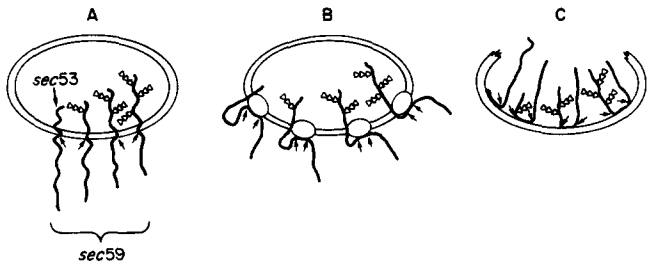

FIGURE 7 Possible orientations of invertase accumulated in *sec53* and *sec59* ER membrane. Oval represents the ER; wavy lines represent the invertase polypeptide; linked triangles represent oligosaccharide chains; arrows indicate the positions of trypsin cleavage that generate protected fragments.

In Fig. 7*A*, the degree of polypeptide penetration is coupled directly to glycosylation. This model predicts that larger glycopeptide fragments will be generated from the higher molecular mass precursors that accumulate in *sec59* compared with *sec53*. Yet the only difference in the pattern is a 9.4-kdalton fragment that is more prominent in *sec53* than in *sec59* (Fig. 5). The failure to observe higher molecular mass fragments in *sec59* may be due to the large number of protected species that could have been produced. Three factors may contribute to this detection problem. First, if after an initial discrete step in penetration, the *sec59* gene product is required continuously during completion of polypeptide penetration, then invertase may be arrested in the mutant at many positions with respect to the ER membrane. Thus, many different trypsin cleavage sites could be exposed adjacent to the cytoplasmic surface of the ER membrane. Second, some of the invertase accumulated in *sec59* may retain the signal peptide; 60- and 63-kdalton forms without carbohydrate were detected (1). If the glycosylated forms also vary in retention of the signal peptide, the number of protected glycopeptides could increase. Third, only 9–10 of the 13 potential glycosylation sites are occupied on mature invertase, and variability in the number of core units has been documented (17, 31). If the glycosylation sites on the protected portion of invertase are variably occupied, the number of trypsin fragments would be increased again. Taken together, these factors suggest that any unique glycopeptide fragment would not be abundant enough to be detected above background.

Alternatively, as depicted in Fig. 7*B*, the immunoreactive fragments could derive from another membrane-protected portion of the invertase polypeptide. Here the protected fragment is associated with an integral membrane protein; it could be bound to the membrane in some other fashion.

In the alternative shown in Figure 7*C*, the isolated membranes are broken and the lumenal surface of the ER is exposed to trypsin, resulting in degradation of all but the membrane-bound portion of invertase. We consider this alternative unlikely. The isolation procedure used to obtain ER membranes from *sec53* and *sec59* has been applied to membranes from *sec* mutants that accumulate active invertase, where it is possible to measure latent enzyme activity. In particular, latent invertase activity is recovered nearly quantitatively in ER vesicles isolated from a mutant that accumulates ER. Latency to sucrose, the invertase substrate, is reduced only slightly by trypsin treatment under conditions that allow cleavage of all the precursor in *sec53* and *sec59* membranes (Fig. 6). Nevertheless, until an independent marker of latency and sidedness is developed for yeast ER, the possibility remains that *sec53* and *sec59* ER are unusually fragile.

Wild-type cells treated with tunicamycin at 37°C also accumulate invertase in a membrane-bound form that can be degraded partially by trypsin (Fig. 6). This suggests that at 37°C, though not at 25°C (Figs. 2 and 3), core glycosylation is essential for import of invertase into the ER. Similarly, export of unglycosylated vesicular stomatitis G protein is temperature sensitive (18). Although a wide range of defects in secretion have been reported to be caused by tunicamycin (32, 33), polypeptide translocation has not yet been implicated. Invertase may represent an extreme case because of the large number of N-linked oligosaccharides per polypeptide chain (9–10 per 60-kdalton polypeptide).

Essentially all the precursors of secreted invertase continue to become membrane associated for as long as 2 h after transfer to 37°C in *sec53* and *sec59*. This represents a significant fraction of the generation time of wild-type cells in this growth medium (3 h). Thus, if accumulated secretory polypeptides occupy ER transport channels, there must be enough to accommodate proteins that would be translocated during more than one-half of a cell generation time. Alternatively, accumulated proteins may become disengaged from such a channel, but remain membrane-bound. Another possibility is that the *sec53* and *sec59* gene products act indirectly to facilitate transport, and do not come in contact with transported proteins.

Recent experiments with reconstituted leader peptidase from *E. coli* suggest that complete translocation of bacterial secreted proteins may require an additional catalyst. Zimmermann and Wickner (34) have found that a precursor of the OmpA protein (located in the outer membrane) is cleaved by sealed liposomes that contain pure leader peptidase on the inner surface of the vesicle. Although the amino terminus of the precursor must cross the vesicle bilayer to be acted upon by the peptidase, the cleaved OmpA product is not retained and instead is found in the supernatant solution after sedimentation of the liposomes. A precursor of the periplasmic maltose-binding protein behaves similarly in this reaction (Y. O. Iwashita and W. Wickner, personal communication).

The isolation of mutants that block translocation after the completion of polypeptide synthesis provides a strong case for membrane proteins involved in the process of penetration. Final resolution of the role of the *SEC53* and *SEC59* gene products will require a polypeptide import reaction with isolated ER vesicles from mutant and wild-type yeast.

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Note added in proof: Mutations in the *SEC53* complementation group have been isolated recently using an [^{3}H]mannose suicide procedure (Huffaker, J., and P. Robbins, *Proc. Natl. Acad. Sci. USA*, in press). Mutations in this gene (which they call *ALG4*) result in the accumulation of a spectrum of lipid-linked oligosaccharides (Man_{1-8}-$GlcNAc_2$), and unglycosylated invertase is produced. Huffaker and Robbins have concluded that *alg4* mutants are not directly defective in oligosacchaide synthesis. These findings are consistent with our conclusions about the role of *SEC53* in polypeptide penetration.

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