Invertase Signal and Mature Sequence Substitutions That Delay Intercompartmental Transport of Active Enzyme

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ABSTRACT The role of structural signals in intercompartmental transport has been addressed by the isolation of yeast invertase (SUC2) mutations that cause intracellular accumulation of active enzyme. Two mutations that delay transport of core-glycosylated invertase, but not acid phosphatase, have been mapped in the 5' coding region of SUC2. Both mutations reduce specifically the transport of invertase to a compartment, presumably in the Golgi body, where outer chain carbohydrate is added. Subsequent transport to the cell surface is not similarly delayed. One mutation (SUC2-s1) converts an ala codon to val at position -1 in the signal peptide; the other (SUC2-s2) changes a thr to an ile at position +64 in the mature protein. Mutation s1 results in about a 50-fold reduced rate of invertase transport to the Golgi body which is attributable to defective signal peptide cleavage. While peptide cleavage normally occurs at an ala-ser bond, the s1 mutant form is processed slowly at the adjacent ser-met position giving rise to mature invertase with an N-terminal met residue. s2 mutant invertase is transported about sevenfold more slowly than normal, with no delay in signal peptide cleavage, and no detectable abnormal physical property of the enzyme. This substitution may interfere with the interaction of invertase and a receptor that facilitates transport to the Golgi body.

The compartmentation of eucaryotic cells implies both specific mechanisms for protein localization and identifying signals that are recognized by the localization apparatus. Protein transport must be targeted not only to distinct organelles but to specific subdivisions within such organelles as the mitochondrion, chloroplast, endoplasmic reticulum (ER), Golgi body, and plasma membrane. This process probably involves a number of unique identifying signals of which only a few have been deciphered. Clearly distinct N-terminal signal peptides direct secretory, mitochondrial, and chloroplast precursors to their respective organelles (1-3); hydrophobic membrane anchoring sequences have been recognized in viral glycoproteins (4, 5) and surface-bound immunoglobulin (6); a cytoplasmic, C-terminal peptide has been implicated in rapid transport of vesicular stomatitis virus G protein from the ER (7); and oligosaccharide phosphorylation triggers the sorting of lysosomal enzymes in mammalian fibroblasts (8-10).

A major question in the mechanism of secretion concerns the role of receptors in mediation of intracellular transport. The evidence for such receptors is entirely circumstantial. Two examples that are consistent with a receptor are that the rate of transport from the ER to the Golgi body varies for different proteins in the same cell (11-13), and that movement of retinol-binding protein from the ER requires retinol, presumably to alter the conformation of the binding protein (14). These cases could equally well be explained by selective negative influences on transport of slowly moving proteins. The challenge in this area is to develop evidence of a more positive nature, such as the identification of a signal or structure required for transport.

One method of defining localization signals in organellar proteins is the isolation of mutations in which these signals are defective so that the altered protein accumulates at some point along the normal transport pathway or is misdirected to some other cellular organelle. The most revealing application of this approach has been in the definition of important
aspects of the signal peptide in secretion of bacterial proteins (15–17). In eucaryotic cells, numerous examples of transport-deficient mutant forms of membrane and secretory proteins have been presented: IgA λ light chain (18), low-density lipoprotein receptor (19), Vaccinia Virus hemagglutinin (20), α1-antitrypsin (21), vesicular stomatitis virus G protein (22–23; for review see reference 24). Defective proteins most often accumulate in the ER. Although some of these mutations may specifically affect a transport signal, it has been difficult to exclude the more trivial possibility that defective proteins are denatured or aggregated.

Retention of full enzyme activity in a secretion-defective mutant protein is a criterion that may be used to distinguish a transport lesion from more general perturbations of protein structure. With this in mind, we have examined invertase, an enzyme that follows the normal secretory pathway in yeast (25). The invertase gene (SUC2) has been cloned and sequenced (26, 27). Transformation of yeast cells with heavily mutagenized plasmid DNA has allowed a large number of SUC2 mutants to be screened for accumulation of active invertase. In this report we describe the isolation, characterization, and specific mapping of two transport-specific mutations.

MATERIALS AND METHODS

Strains, Growth Conditions, and Materials: Saccharomyces cerevisiae strains SEY1201, SEY1202, and SEY5188 (all suc2-29), and SEY5186 (SUC2, suc1-52), were described previously (28, 29). ISY1-22A (ura3-52, leu3-3, his4-519, suc2-29, pho80-2) was used as a standard genetic technique.

pRB58, a Yep24-derivative 2 μ plasmid (30) containing the SUC2 gene, was obtained from M. Carlson (Columbia University) and has been described elsewhere (26). Plasmids pSEY125, pSEY124, and pSEY122, which carry SUC2-lacZ fusions, have also been described elsewhere (29). pSEY8 was derived from pUC8 (31) and also contains a 2 μ DNA and the URA3 gene (30).

YPD medium contained 1% Bacto-Yeast extract (Difco Laboratories Inc., Detroit, MI), 2% Bacto-peptone (Difco Laboratories Inc.), and 2% glucose. Wickerham's minimal medium (32) was used with various amounts of glucose or raffinose as a carbon source. For low sulfate medium, chloride salts replaced sulfate salts, and ammonium sulfate was added to the desired concentration.

The absorbance of dilute cell suspensions was measured in a 1-cm cuvette at 600 nm in a Zeiss PMQII spectrophotometer; 1 OD600 unit of cells corresponds to 0.15 mg dry wt. Liquid cultures were grown in flasks or tubes with agitation, and experiments were initiated with exponentially growing cells at an OD600 of 0.5–4.

Other reagents were obtained as indicated: glucose oxidase, peroxidase, o-dianisidine, and 5-bromo-4-chloro-3-indolyl phosphate, pH 7.0, 200 mM sodium chloride) at ~800 OD600 units of cells/ml and lysed by agitation for 5 min with glass beads (0.3–0.5 mm) in a Bead-Beater (Biospec Products, Bartlesville, OK). The extract was then adjusted to 1% SDS and heated in a boiling water bath for 6 min. A soluble fraction (30–40 mg protein/ml) was obtained by centrifugation for 20 min at 12,000 g.

Mutagenesis and Mutant Screening: pRB58 plasmid DNA, purified for C6C6 density sedimentation (data not shown), was digested into fragments of 0.4 M hydrogen peroxide, 0.05 M potassium phosphate, pH 6.0, at a DNA concentration of 125 μg/ml. After 60 min at 75°C, samples were chilled, and the DNA was precipitated with ethanol, resuspended in 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, and precipitated with ethanol an additional two times. Yeast strain SEY2102 was transformed with mutagenized DNA by the lithium acetate procedure (35). Each plate contained minimal medium without uracil, was spread with cells exposed to 2 μg of DNA.

Individual transformants were spotted onto selective plates (minimal medium without uracil) and screened for invertase secretion by one or both of the two methods. The first screen involved a qualitative assay of invertase on a filter paper disk. Plates of 100 transformants were stamped in duplicate onto plates containing VP medium (no glucose) and incubated at room temperature for 3–4 h during which time invertase synthesis was derepressed. One of each pair of plates was inverted over a chloroform-soaked paper towel for 10 min. Whatman No. 1 filter disks (Whatman Laboratory Products Inc., Clifton, NJ) were bathed in a 2-mI solution that contained the invertase assay reagents: 0.1 M sodium acetate, pH 5.1, 0.125 M sucrose, 0.4 M N-ethylmaleimide, 10 μg/ml peroxidase, 0.6 mg/ml o-dianisidine, and 0.05 mg/ml glucose oxidase. Chloroform-treated and untreated copies of the transformants were stamped onto filter disks. Within a few minutes at room temperate, a pink color developed over most of the patches. This procedure was used to identify mutant suc2Δ, which showed more intense staining in the chloroform-treated replica, but did not give sufficient resolution to detect the other mutants.

The other screen procedure involved analysis of intracellular invertase activity by electrophoresis on nondenaturing polyacrylamide gels. Cultures (5 ml) of individual transformants were grown to mid-logarithmic phase in minimal medium + 5% glucose. A mixture of 1 OD600 unit of cells from each of five cultures were centrifuged, washed with water, and incubated in minimal medium and 0.1% glucose for 3 h at 25°C. Cells were then washed with 10 mM sodium azide, and 1 OD600 was resuspended in 0.1 ml of 1 M sorbitol, 50 mM potassium phosphate, pH 7.5, 10 mM sodium azide, 80 mM 2-mercaptoethanol, and 50 U of leucocase. After 60 min at 30°C, spheroplasts that sedimented at 3000 g for 10 min by resuspension in 50 mM potassium phosphate, pH 7.5, 10 mM sodium azide, 0.1% Triton X-100. An aliquot corresponding to 0.15 OD600 unit of cells was electrophoresed on a 5% polyacrylamide slab gel using a system described by Meyer and Matile (36) except without SDS. Invertase was localized in the gel, after incubation at 25°C for 20–60 min in 0.1 M sodium acetate (pH 5.0), 0.1 M sucrose, by the method of Gabriel and Wang (37). Pools containing mixtures of wild-type and suc2Δ transforms showed primarily the rapidly and discretely migrating cytoplasmic invertase with faint staining in the region that contained glycosylated forms of invertase. In pools that showed more intense staining in this latter region, individual members were screened directly for invertase secretion and accumulation by the assay of Goldstein and Lampen (33) with minor modifications (38). A unit of invertase will release 1 μmol of glucose from sucrose per minute at 25°C.

Labeling, Immunoprecipitation, and Endo H Treatment: Cells were grown to mid-logarithmic phase in minimal medium + 5% glucose and 0.1 mM ammonium sulfate. 1 OD600 unit of cells was centrifuged, washed with water, and resuspended in fresh minimal medium + 0.1% glucose and no sulfate at 1 to 2 OD600 units of cells/ml. These conditions allowed derepression of invertase synthesis. After 20 min at 25°C, 250 μCi of carrier-free H235SO4 was added and cultures were incubated an additional 30 min. Labeled cells were washed and converted to spheroplasts as described in the previous section. Spheroplasts were centrifuged, the supernatant fractions were adjusted to 0.5% SDS, and the pellets were resuspended in 50 μl of 1% SDS. Samples were heated in boiling water for 3 min, diluted to 1 ml with PBS and 2% Triton X-100, and treated with 25–50 μl of IgG Sorb, prepared as described by the manufacturer, for 30 min at 0°C. IgG Sorb was removed by centrifugation at 12,000 g for 20 min and supernatant fractions were transferred to new microfuge tubes. Antibody was added (inversion serum, 4 μl/OD600 unit cell equivalent; acid phosphatase serum, 4 μl/OD600 unit cell equivalent for spheroplasts, and 100 μl/OD600 unit cell equivalent for spheroplast supernatant fractions) and samples were incubated at 0°C for 2 h. Inversion immunoprecipitation samples were supplemented with 125 μl of (4–5 mg protein) of nonradioactive extract from strain SEY1201 and served to control for the presence of unrelated proteins. IgG Sorb was added at 10–20 μl per 1 μl of serum, and immune complexes formed during 30 min at 0°C were sedimented at 12,000 g for 1 min. Complexes were washed twice with 0.5 ml of 2 M urea, 0.2 M sodium chloride, 0.1 M Tris-HCl, pH 7.5, 1% Triton X-100, and then twice with 0.5 ml of 1% 2-mercaptoethanol. Final pellets were resuspended in 25–50 μl of sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 2%-

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mercaptoethanol (0.01% b-mercaptoethanol) and heated in boiling water for 3 min. After centrifugation in a microfuge for 5 min, the supernatant fractions were subjected to gel electrophoresis on 6% SDS-polyacrylamide slab gels according to the procedure of Laemmli (39). Gels were fixed, in some cases prepared for fluorography with ENHANCE, dried, and allowed to expose Kodak X-Omat AR film at -70°C. Autoradiograms were quantified by scanning with an E-C Apparatus Corp. (St. Petersburg, FL) ECS910 densitometer trace.

Modifications of the standard procedure were used in certain experiments. In the kinetic analysis, cells were incubated with H3P04 at 1 OD600 unit/ml for 5 min (or 10 min for labeling of acid phosphatase). Cultures were then adjusted to 0.5% glucose, 1 mM ammonium sulfate, 100 μg/ml cycloheximide, to initiate the chase phase. Aliquots of 1 OD600 unit of cells were taken at various times and added to chilled tubes that contained 10 μl of 1 M sodium azide. Cells were washed once with cold 10 mM sodium azide and held at 0°C until the conclusion of the experiment. In all experiments, the experiment in which acid phosphatase was assayed, samples were split and one-half of each was treated with the respective antiserum. For treatment with endo H, aliquots of immunoprecipitated invertase, after solubilization in sample buffer and separation from IgG Sorb, were diluted twofold and adjusted to 0.1 mg/ml BSA, 0.1 M sodium acetate, pH 5.1. Samples were incubated with 0.5 μl of endo H overnight at 37°C. After being heated in boiling water, samples were subjected to SDS gel electrophoresis.

N-Terminal Analysis of Mutant and Wild-type Invertase: For N-terminal analysis of secreted invertase, strain SEY2102 containing pRB83 with either SUC2 or the sl mutant was derepressed for invertase synthesis (4 OD600 units of cells) in 2 ml of minimal medium with no sulfate and 0.1% glucose. After 10 min at 25°C, 2 ml of H3P04 were added to each sample for 60 min at 25°C, and a chase period was initiated as described earlier for the kinetic analysis. Cells were centrifuged after a 60-min chase period, washed with 10 mM sodium azide, and converted to spheroplasts. Invertase in the E fraction was immunoprecipitated as described earlier, using anti-invertase antibody that had been affinity-purified by adsorption to and elution from cytoplasmic invertase-conjugated to Sepharose 4B. The precipitates were solubilized in 30 μl of sample buffer and separated from IgG Sorb by centrifugation; 1 μl of each was subjected to SDS gel electrophoresis to confirm the purity of the labeled samples. The remaining samples were treated with 1 ml acetic acid/triethylamine/acetic acid (90:5:5) and then three times with acetone. Each extraction was incubated for 20 min at 0°C and then centrifuged at 12,000 g for 10 min. The final pellets were resuspended in 50 μl of 10 mM ammonium bicarbonate, 0.02% SDS. Samples were subjected to sequential Edman degradation using an Applied Biosystems (Foster City, CA) 470A gas-phase protein sequencer. Material released in each of the first 15 cycles was measured for [35S]cysteine.

For N-terminal analysis of ER-localized invertase, 5 OD600 units of SEY1588 (sec18) containing pRB83 with either wild-type or sl mutant SUC2 genes were transferred to 1 ml of minimal medium with 0.1% glucose, no sulfate, and 4 μM leucine. After 10 min at 25°C, 250 μg of [3H]leucine and 2 μCi H3P04 were added to each sample for 60 min at 37°C, washed with 10 mM sodium azide, and converted to spheroplasts. Invertase in the E fraction was immunoprecipitated as described earlier, using anti-invertase antibody that had been affinity-purified by adsorption to and elution from cytoplasmic invertase-conjugated to Sepharose 4B. The precipitates were solubilized in 30 μl of sample buffer and separated from IgG Sorb by centrifugation; 1 μl of each was subjected to SDS gel electrophoresis to confirm the purity of the labeled samples. The remaining samples were treated with 1 ml acetic acid/triethylamine/acetic acid (90:5:5) and then three times with acetone. Each extraction was incubated for 20 min at 0°C and then centrifuged at 12,000 g for 10 min. The final pellets were resuspended in 50 μl of 10 mM ammonium bicarbonate, 0.02% SDS. Samples were subjected to sequential Edman degradation using an Applied Biosystems (Foster City, CA) 470A gas-phase protein sequencer. Protein released in each of the first 15 cycles was measured for [35S]cysteine.

Recombinant DNA and Marker Rescue Mapping: Plasmid purification, agarose gel electrophoresis, transformation of bacteria, and other DNA manipulations were performed by standard methods (42).

Wild-type, sl, and s2 SUC2 genes were subcloned into YIp5 (30) by ligation of a 4.5 base pair Xhol to Clal fragment of the SUC2 insert into YIp5 cut with SalI and Clal. Uncut YIp5 containing wild-type and mutant SUC2 genes were then transformed into SEY2102. URA+ transformants resulting from integration of YIp5 into the genome were picked and assayed for invertase secretion.

For construction of mutant- and wild-type hybrid genes, SUC2 was first subcloned by inserting the HindIII fragment of pRB85 into the unique HindIII site of pSEY8 (see Fig. 6). The 800 base EcoRI-BamHI fragment of this pSEY8 construct was then replaced with the 1,600 base EcoRI-BamHI fragment of SUC2, s2, or s3 from mutant pRB88 plasmids. In each case, then, each of the 5' half of a mutant gene and the 3' half of wild-type SUC2.

For marker rescue mapping, SUC2-s2, -s3, and -s3 HindIII fragments were subcloned into the pSEY8 HindIII cloning site, and the resulting plasmids were introduced into yeast strain SEY2102 (a sec2-29). SEY2102 (a sec2-29) was transformed with the SUC3-lacZ fusion vectors pSEY125, pSEY124, and pSEY129. Strains were grown on YPD plates, and mating patches were resuspended in water and spread on minimal medium plates 2% sucrose, 0.02 mg/ml ergosterol, 0.25 mg/ml oleate, 2.5 mg/ml Tergitol Nonident P-40, and 20 μg/ml uracil. Plates were incubated at 30°C in an anaerobic jar using the BBL-Gas Pak anaerobic system. These conditions allowed growth only of SUC+ recombinants that were picked after 5-6 d. SUC+ colonies were streaked on fresh plates and grown selectively for an additional 4 d. The number of recombinant colonies represented the number of original recombinants in the liquid medium, and secretion and accumulation of invertase were assessed as before.

DNA Sequencing: Preparations of each of the mutant SUC2 plasmids were digested with BamHI and HindIII. The 776 base fragment that contained the 5' half of each mutant SUC2 gene was purified by gel electrophoresis in and extraction from low molten temperature agarose (43). M13 mp8 or M13 mp9 RF (replicative form) DNA was digested with M13 mp9 RF (replicative form) DNA was digested with HindIII, Sall, or the sl mutant was derepressed for invertase synthesis (4 OD600 units of cells) in 2 ml of minimal medium with no sulfate and 0.1% glucose. After 10 min at 25°C, 250 μg of [3H]leucine and 2 mCi 35SO4 were added to each sample for 60 min at 37°C, washed with 10 mM sodium azide, and converted to spheroplasts. Invertase in the E fraction was immunoprecipitated as described earlier, using anti-invertase antibody that had been affinity-purified by adsorption to and elution from cytoplasmic invertase-conjugated to Sepharose 4B. The precipitates were solubilized in 30 μl of sample buffer and separated from IgG Sorb by centrifugation; 1 μl of each was subjected to SDS gel electrophoresis to confirm the purity of the labeled samples. The remaining samples were treated with 1 ml acetic acid/triethylamine/acetic acid (90:5:5) and then three times with acetone. Each extraction was incubated for 20 min at 0°C and then centrifuged at 12,000 g for 10 min. The final pellets were resuspended in 50 μl of 10 mM ammonium bicarbonate, 0.02% SDS. Samples were subjected to sequential Edman degradation using an Applied Biosystems (Foster City, CA) 470A gas-phase protein sequencer. Protein released in each of the first 15 cycles was measured for [35S]cysteine.

RESULTS

Mutagenesis of Cloned SUC2 Gene

A 2 μ yeast plasmid containing the SUC2 gene and a selectable marker gene (URA3) was mutagenized in vitro with hydroxylamine which produces C to T transitions. Mutant DNA was introduced by transformation into a ura3 strain that contained a deletion in the SUC2 gene (28). Hydroxylamine treatment was adjusted so that 10-15% of the plasmids that were recovered by transformation contained SUC2 mutations resulting in no active invertase. The efficiency of transformation by DNA mutagenized at this level was reduced twofold for yeast and 104-fold for E. coli.

In a sampling of 60 independent suc- mutants, 31 failed to make any invertase immunoreactive protein, which, in most cases, was due to major rearrangements in the plasmid DNA. Of the remaining 29 mutants in which immunoreactive ma...
doplasmic reticulum (46). These mutations may produce denatured forms of invertase that can not be transported from the ER.

To identify mutations that affect transport but not correct folding of the invertase polypeptide, we screened 6,000 independent transformants by procedures that detect intracellular accumulation of active invertase. Four were found that fit this criterion, and the extent of invertase accumulation is shown in Table I. Cells transformed with the normal or mutant (SUC2-s1 to -s4) plasmids were derepressed for invertase synthesis, converted to spheroplasts, and the released enzyme (not shown). Mutant s4 was eliminated from further consideration because the low amount of periplasmic invertase derepressed under steady-state conditions of growth, whereas the amount of invertase dropped 10-fold, the extent of accumulation was unaffected (Table I, lines 9–11). These results also showed that the mutations were linked to SUC2.

**Mutants Defective in Transport of Core-glycosylated Invertase**

Invertase transport intermediates have been identified in pleiotropic sec mutants that are blocked at successive stages in the secretory pathway. Mutants such as sec18, in which transport from the ER to the Golgi body is blocked, represent the earliest stage where an active secretory form of invertase is detected. SDS gel electrophoresis of invertase accumulated in sec18 reveals several discrete species of around 80 kd, corresponding to core-glycosylated molecules. Subsequent oligosaccharide modifications in the Golgi body produce heterogeneous, low electrophoretic mobility forms of invertase (46).

To identify the site of accumulation of mutant invertase, we labeled cells (wild type and sec18) transformed with plasmids carrying wild-type, SUC2-s1, -s2, or -s3 mutant genes for 30 min with 35SO42-, converted them to spheroplasts, and evaluated the periplasmic (external, E) and intracellular (I) fractions by immune precipitation and SDS gel electrophoresis of spheroplast extracts on a nondenaturing gel system. Mutants s1, s2, and s3 produced normal amounts of this enzyme (not shown). Mutant s4 was eliminated from further consideration because the low amount of periplasmic invertase produced was less stable and cytoplasmic invertase was inactive.

**Table 1**

| Line | Plasmid/SUC2 type | Derepressed intracellular invertase* | Periplasmic invertase | Total activity | Fold accumulation† |
|------|------------------|-----------------------------------|----------------------|----------------|-------------------|
| 1    | pRB58/SUC2       | 0.40                              | 1.71                 | 100            | 1                 |
| 2    | pRB58/SUC2-s1    | 1.96                              | 0.28                 | 106            | 30                |
| 3    | pRB58/SUC2-s2    | 1.12                              | 0.76                 | 89             | 6.7               |
| 4    | pRB58/SUC2-s3    | 1.13                              | 0.87                 | 95             | 5.7               |
| 5    | pRB58/SUC2-s4    | 0.52                              | 0.43                 | 45             | 5.2               |
| 6    | pRB58/SUC2       | 0.04                              | 0.77                 | 100            | 1                 |
| 7    | pRB58/SUC2-s1    | 0.60                              | 0.35                 | 117            | 34                |
| 8    | pRB58/SUC2-s2    | 0.16                              | 0.53                 | 85             | 6                 |
| 9    | YIp5/SUC2        | 0.03                              | 0.29                 | 100            | 1                 |
| 10   | YIp5/SUC2-s1     | 0.15                              | 0.05                 | 63             | 33                |
| 11   | YIp5/SUC2-s2     | 0.27                              | 0.22                 | 150            | 12                |
| 12   | pRB58/SUC-s1 Eco-Bam hybrid | 0.74                              | 0.12                 | 62             | 36                |
| 13   | pRB58/SUC2-s2 Eco-Bam hybrid | 0.71                              | 0.90                 | 116            | 6                 |
| 14   | pRB58/SUC2-s3 Eco-Bam hybrid | 0.55                              | 0.83                 | 99             | 5.1               |

* SEY2102 was transformed with the indicated plasmids. Except in the experiment in lines 6–8, cells were grown to mid-log phase in selective minimal medium and 5% glucose, washed, and derepressed for 3–4 h at 25°C in minimal medium and 0.1% glucose. Cells were converted to spheroplasts and centrifuged, and the supernatant (periplasmic) and pellet (intracellular) fractions were assayed for invertase activity. A constant background of cytoplasmic invertase activity was subtracted to give the derepressed intracellular level. Cells in the experiment in lines 6–8 were grown in minimal medium and 2% raffinose.

† The ratio of derepressed intracellular to periplasmic activity for mutant SUC2 alleles divided by the ratio for wild-type SUC2.

‡ Results of a separate experiment with values normalized to assays of pRB58/SUC2 done in parallel.
resis. After this labeling period in wild-type cells containing pRB58, the cytoplasmic invertase and some of the ER transit form were found in the I fraction, while much of the invertase was highly glycosylated and in the E fraction (Fig. 1). The ER transit forms accumulated in sec18 were similar to those found in wild-type cells. SUC2 s1, s2, and s3 produced nearly exclusively ER transit forms of invertase. Mutant s1 (and s2, not shown) showed the same behavior when integrated into the chromosome (single copy). Unlike the pleiotropic sec mutants, the SUC2 transport mutants were blocked to the same extent at 25°C and 37°C (not shown). Hence, by comparison with the behavior of sec18, the SUC2 mutants appear to be nonconditionally defective in transport from the ER, or possibly from a compartment between the ER and the site of outer chain carbohydrate addition.

In experiments with longer radiolabeling periods, highly glycosylated, secreted invertase was detected in cells that contained the s1, s2, and s3 mutant alleles. The possibility that these mutants were not absolutely defective in transport allowed additional definition of the stage in the pathway and of the specificity of the defects.

If the transport-defective invertase is delayed in the secretory pathway before or concurrent with the SEC18 dependent step, then invertase that accumulates at 25°C will not progress beyond the sec18 block at 37°C. On the other hand, if mutant invertase is delayed beyond the SEC18 step, progress will not be hindered in sec18 cells at 37°C. sec18 cells, transformed with a plasmid carrying SUC2-s3, were labeled with 35SO42- for 10 min at 24°C (sec permissive temperature). Cultured aliquots were subsequently incubated for 30 and 60 min at 24°C and 37°C (sec nonpermissive temperature) in the presence of cycloheximide. Intracellular and external invertase was examined by immunoprecipitation (Fig. 2). After the pulse, only core-glycosylated invertase in the I fraction was observed. With increasing chase time at 24°C, heterogeneous glycosylated material was detected in the E fraction, with a corresponding decline in core-glycosylated protein in the I fraction. No further glycosylation or secretion was seen when the chase was conducted at 37°C. This pattern was qualitatively similar in mutants s1 and s2, suggesting that all the invertase defects delay transport before or at the SEC18 step. Control experiments with wild-type cells containing mutant invertase showed secretion of about one-half of the s1 invertase, and all of the s2 mutant protein, during the 60-min chase at 37°C.

Differences among the invertase mutants were uncovered by a more detailed analysis of the kinetics of secretion at 24°C. Cells that carried plasmids with wild-type, s1, or s2 invertase were pulse-labeled for 5 min. At various times during subsequent incubation in the presence of cycloheximide, samples were withdrawn and the distribution of immunoreactive forms of invertase was evaluated. Fig. 3 shows the percentage of radiolabeled invertase (open symbols, core glycosylated; closed symbols, heterogeneous forms) that remained intracellular at the indicated times of chase. In the pulse label, 60% of the wild-type invertase was core glycosylated and intracellular, whereas the highly glycosylated material was equally divided between intracellular and secreted. Conversion of core- to highly-glycosylated invertase occurred with a t1/2 of ~1.5 min for the wild-type, ~75 min for mutant s1, and ~10 min for mutant s2 (same as s3, not shown). A semilogarithmic plot of the data for the mutants revealed a single kinetic constant for conversion of invertase. In each case, the total amount of labeled invertase remained constant during the chase period. A transient increase in highly glycosylated intracellular invertase was seen early in the chase period of wild-type protein, while mutant s1 and s2 showed negligible intracellular levels of this form. These results suggest that mutants s1 and s2 are distinct, and that secretion is specifically delayed in the ER with no observable effect on subsequent transport from the Golgi body.

Additional evidence for the specificity of the invertase mutations came from an analysis of acid phosphatase secretion and growth rates of strains that contained the mutant SUC2 plasmids. A strain that carries the pho80-2 mutation, and therefore constitutively makes and secretes acid phosphatase (47), was transformed with wild-type or mutant SUC2 plasmids. Cells were derepressed for invertase expression and labeled for 10 min at 25°C, and samples were withdrawn at intervals during a chase in the presence of cycloheximide. The distribution of radiolabeled forms of acid phosphatase was

**Figures 1** Immunoprecipitation and electrophoresis of wild-type and mutant invertases. SEY2102 cells transformed with pRB58 containing either wild-type or mutant SUC2 genes and SEYS188 (sec18) containing pRB58 were derepressed for invertase expression and labeled with 35SO42- for 30 min. Samples were separated into spheroplast pellet (I) and spheroplast supernatant (E) fractions and immunoprecipitated with invertase antiserum. Immunoprecipitates were analyzed by SDS PAGE and autoradiography. mature, highly glycosylated invertase; ER, core-glycosylated invertase characteristic of endoplasmic reticulum forms; cyto, cytoplasmic invertase.
of the chase period by addition times, separated into spheroplast. Aliquots were taken at various times, separated into spheroplast pellet (f) and spheroplast supernatant (E) fractions, and immunoprecipitated with invertase antiserum. Immunoprecipitates were analyzed by SDS PAGE and autoradiography.

**FIGURE 2** Secretion of s3 mutant invertase blocked in sec18 at 37°C. SEY5188 (sec18) cells transformed with pRB58 that contained SUC2-s3 were derepressed for invertase expression and labeled with 35SO42- for 10 min at 25°C. Immediately after initiation of the chase period by addition of glucose, unlabeled sulfate, and cycloheximide, an aliquot of the culture was transferred to 37°C and the rest was kept at 25°C. Aliquots were taken at various times, separated into spheroplast pellet and supernatant fractions, and immunoprecipitated with invertase antiserum. Immunoprecipitates were analyzed by SDS PAGE and autoradiography.

**FIGURE 3** Kinetics of wild-type and mutant invertase secretion. SEY2102 cells that contained either the wild-type or s2 mutant SUC2 genes were derepressed for invertase expression and labeled for 5 min with 35SO42-. Chase was initiated with the addition of glucose, unlabeled sulfate, and cycloheximide. Aliquots were taken at various times, separated into spheroplast pellet and supernatant fractions, and immunoprecipitated with invertase antiserum. Immunoprecipitates were subjected to SDS PAGE and fluorography. Cross-reacting material was quantified by densitometric scanning. Evaluated by immunoprecipitation and SDS gel electrophoresis. As with invertase, acid phosphatase undergoes a core-to-highly-glycosylated conversion after transport to the Golgi body (46). Core-glycosylated acid phosphatase was converted and secreted at the same rate in cells that were producing wild-type or mutant invertase (not shown). In the same samples, the pattern of invertase glycosylation and secretion was identical to that seen in Fig. 3. Furthermore, the invertase mutants did not interfere with any essential aspect of growth; doubling times in derepressing media were the same for cells carrying wild-type or SUC2 mutant plasmids.

**Physical Properties of the Mutant Invertases**

Delayed secretion of mutant invertase could be accounted for by aberrant subunit interaction or aggregation within the lumen of the ER. This possibility was tested by native gel electrophoresis of invertase released from membranes by treatment with Triton X-100. Secreted invertase, detected with an activity stain, migrates as a heterogeneous mixture, whereas enzyme accumulated in the ER (such as in sec18) displays four discrete species which appear to be multimeric forms of the active dimer (46). For comparison, wild-type and sec18 cells containing SUC2, SUC2-s1 (on a multicopy plasmid or integrated), -s2, or -s3 were derepressed for invertase synthesis and fractionated as before. Fig. 4 shows the pattern described above for wild-type and sec18 cells. The intracellular enzyme produced by mutants s2 and s3 migrated as did invertase in sec18. Mutant s1, however, appeared aggregated with a heterogeneous and low electrophoretic mobility. A pattern more like that of sec18 was seen with cells that contained a single copy of mutant s1, although most of the invertase was concentrated in the lowest mobility forms.

While Triton X-100 quantitatively released the ER-accumulated forms of invertase, at least one ER integral membrane protein, NADPH-cytochrome c reductase, was also solubilized. To address the issue of invertase solubility in the ER lumen, saponin, a detergent that permeabilizes but does not solubilize the ER membrane, was used to examine release of invertase activity from the membrane fraction. Spheroplasts made from cells (wild type or sec18) that had accumulated normal or mutant invertase were osmotically lysed under conditions that retained invertase in a sedimentable, presumably ER-bounded form. Extracts were mixed with various amounts of saponin at 0°C, and the amounts of sedimentable protein, NADPH-cytochrome c reductase, and invertase were assessed. Fig. 5 shows that very little protein (top) and no NADPH cytochrome c reductase (middle) were solubilized by saponin concentrations as high as 0.6 mg/mg protein. Wild-type invertase accumulated in sec18 and s2 mutant invertase were readily released (70-80% solubilized; Fig. 5, bottom) by saponin at 0.2–0.3 mg/mg protein. In contrast, only ~35% of the s1 mutant invertase was released by saponin. S1 mutant enzyme that resisted saponin extraction was solubilized with 0.1% Triton X-100 and compared with the saponin-released invertase by native gel electrophoresis. Heterogeneous, low-mobility invertase was enriched in the saponin pellet fraction and discrete, higher-mobility forms were recovered in the saponin soluble fraction (not shown). Hence, by the native gel electrophoresis and saponin solubility criteria, s1 mutant invertase appears to be aggregated and to some extent associated with the ER membrane, whereas the
FIGURE 4 Nondenaturing gel electrophoresis of wild-type and mutant invertase. SEY2102 cells containing either wild-type or mutant SUC2 genes and SEY5188 (sec18) cells containing the wild-type SUC2 gene were derepressed for invertase expression for 3 h and separated into spheroplast pellet (I) and spheroplast supernatant (E) fractions. Samples were subjected to nondenaturing gel electrophoresis, and the gel was stained for invertase activity.

FIGURE 5 Saponin solubilization of accumulated invertase. SEY2102 cells that contained the mutant SUC2 genes and SEY5188 (sec18) cells with the wild-type SUC2 gene were derepressed for invertase expression and converted to spheroplasts. Sedimented spheroplasts were lysed by osmotic shock. Aliquots were treated with different saponin concentrations and centrifuged at 133,000 g. Pellet and supernatant fractions were assayed for invertase activity (bottom), NADPH-cytochrome c reductase activity (middle), and total protein (top). Values represent the fraction sedimentable relative to the no detergent control. O, sec18; ●, SUC2-s1; Δ, SUC2-s2.

Since the SUC2 mutants were derived from DNA that was heavily mutagenized, it was likely that the isolates contained multiple mutations. For this reason, it was essential to localize the phenotypically important mutations by genetic mapping.

We localized the transport-deficient mutations to the 5' half of SUC2 by subcloning a 1,600 base pair EcoRI-BamHI fragment from the mutant DNA, containing about 770 bases of the 5' coding region, into a vector that contained the 3' half of wild-type SUC2. Donor and recipient plasmids are diagrammed in Fig. 6. Yeast transformants that contained the hybrid constructs showed the same invertase accumulation phenotype of the parents (Table I, lines 12-14). The accumulated material was examined by nondenaturing and SDS gel electrophoresis, and with one minor exception, the original phenotypes were reproduced. In the original sl isolate, cytoplasmic invertase migrated more slowly than normal on a nondenaturing gel (Fig. 4). The EcoRI-BamHI hybrid of mutant sl produced a normal cytoplasmic invertase (not shown), suggesting that the 3' half of the original isolate contained at least one additional mutation that had no detectable influence on invertase transport.

To localize the transport mutations more precisely within the 5' coding region, we devised a form of marker rescue recombinant analysis. HindIII fragments from plasmids carrying SUC2 mutants -s1, -s2, and -s3 were subcloned into the HindIII site of pSEY8 (Fig. 6). These constructs were missing the 5' regulatory sequences and the first 10 bases coding for the secreted form of invertase. Cells transformed with these plasmids were mated with cells carrying another plasmid that contained one of a series of gene fusions in which a portion of the wild-type SUC2 5' regulatory and coding sequence was fused to a nearly full-length fragment of the E. coli lacZ gene (29). Neither construct allowed invertase synthesis; consequently, anaerobic growth of diploid strains on sucrose provided a selection for recombinants that generated functional invertase. Since the presence of even the s1 mutant invertase allowed growth on sucrose, wild-type and transport-deficient recombinants were generated. Wild-type recombinants could be generated only if the region containing the transport mu-

s2 mutant enzyme is indistinguishable from normal invertase accumulated in the ER lumen. s2 and s3 mutant enzymes were identical in these characteristics. In other experiments, the mutant invertases had heat stability properties similar to those of the wild-type enzyme.
tation was represented on the SUC2 lacZ fusion. The ratio of wild-type and mutant recombinants was an approximate measure of the position of the mutation within the region of homology. This point is illustrated in Fig. 7: Mutations in region A would give mainly wild-type recombinants, in B would yield a mixture of mutant and wild-type, and in C would produce only mutant recombinants. Fig. 8 presents data on the percentage of wild-type recombinants generated in crosses between each mutant and hybrid genes that contained 150, 600, or 800 bases of SUC2. Mutant s1 showed a high percentage of wild-type recombinants even with the smallest SUC2 fusion tested. Mutants s2 and s3 showed wild-type recombinants only with the next larger SUC2 fusion. The possibility that transport mutations are 5′ to the HindIII site in the coding sequence is eliminated by these data. Approximate map positions for these mutations are indicated in Fig. 8. Additional mutations required for the transport defect could reside between the marker rescue site and the BamH1 site in the middle of the gene.

The transport mutations were defined precisely by DNA sequence analysis of the 776 base HindIII-BamH1 fragment from each mutant plasmid. SUC2-s1 contained two mutations in this region. One silent mutation resulted in exchange of the codons at amino acid position 150 (TTC to TTT). The important mutation produced a change in the final codon of the signal peptide from Ala to Val (GCA to GTA). Although SUC2-s2 and -s3 mutations were derived from separate samples of mutagenized DNA, they both contained the same single substitution resulting in ile in place of thr (ACT to ATT) at position +64 in mature invertase. Two segments of the invertase protein sequence in the area of mutations s1 and s2 are shown in Fig. 9. A close correspondence was found between the map position determined by marker rescue recombinant analysis and DNA sequencing of the mutations.

Signal Peptide Processing of Mutant Invertase

Secreted invertase is processed from a precursor that contains a 19 amino acid signal peptide (48). Since valine is not found on the N-terminal side of signal peptide cleavage sites (49), SUC2-s1 mutant invertase could be defective in cleavage by signal peptidase. Retention of the signal peptide on mutant invertase was assessed by treatment of accumulated and secreted material with endo H. sec18 and wild-type cells containing the SUC2, -s1, or -s2 plasmids were radiolabeled with...
Figure 8 Mutations map to a region of ≤250 base pairs at the 5' end of the SUC2 gene. SEY2102 cells containing 5' truncated constructs of the mutant SUC2 genes were mated with SEY2101 containing three different size classes of SUC2-lacZ fusions. pSEY125 contained 150 bases, pSEY124 contained 600 bases, and pSEY122 contained 800 bases of SUC2 coding sequence. The table gives the percentage of wild-type recombinants from each cross. The number in parenthesis is the number of recombinants analyzed for each cross. The sketch shows the approximate positions of the mutations predicted by the mapping data.

|       | SUC2-s1 | SUC2-s2 | SUC2-s3 |
|-------|---------|---------|---------|
| pSEY125 (150 bp) | 80% wt (40) | 0% wt (47) | 0% wt (36) |
| pSEY124 (600 bp) | 100% wt (12) | ~90% wt (28) | ~70% wt (30) |
| pSEY122 (800 bp) | 100% wt (5) | 100% wt (2) | N.D. |

Figure 9 Amino acid changes in SUC2 transport mutants.

35SO42- for 30 min and converted to spheroplasts, and the I and E fractions were treated with invertase antibody. Samples of the immunoprecipitates were treated with endo H under conditions where all the oligosaccharides are cleaved from invertase. Untreated and deglycosylated invertase samples were compared by SDS gel electrophoresis. Endo H treatment produced a form with an apparent Mr of 58,500 for normal invertase accumulated in sec18, and s2 invertase in normal cells (Fig. 10). The difference of ~2.5 kd between this species and the cytoplasmic invertase was expected from the 10-12 GlcNAc residues that remain after endo H treatment. Two forms of deglycosylated s1 invertase of approximately equal abundance were seen in the I fraction. One was the 58.5-kd species and the other was a 61-kd form corresponding to an increment expected for retention of the signal peptide. The same 2.5-kd size difference was seen in a comparison of unglycosylated s1 and wild-type invertase produced in tunicamycin-treated cells (not shown). By this criterion, signal peptide processing was normal for mutant s2 but defective for s1 mutant invertase.

After a chase period of 2 h, invertase secreted in mutant s1 was in the usual highly glycosylated form. Only the 58.5-kd species was detected when this material was treated with endo H (Fig. 10). Since the immunoreactive invertase was recovered in good yield after the chase period, some of the glycosylated precursor must have been proteolytically processed en route to the periplasm.

To confirm the accumulation of unprocessed invertase, and to determine the site of cleavage of the mutant protein, we performed sequential Edman degradation of 35SO42-[3H]leucine double-labeled proteins. Wild-type forms of invertase accumulated in sec18, or secreted in a wild-type strain, were removed from SDS gels of samples that had been treated with endo H. Table II shows that 35S was released in the second cycle of Edman degradation and 3H was released in cycle 11. This was expected from the location of met and leu in mature invertase at position 2 and 11, respectively. sl invertase that accumulated in a wild-type cell in the apparently unprocessed and processed forms were compared with the mutant secreted form by the same analysis. As expected from the sequence of the gene, 35S was released from the unprocessed form in cycle 1, and 3H in cycles 2, 3, 7, 9, and 10. In contrast to wild-type
Figure 10  Endo H treatment of wild-type and mutant invertases.
SEY5188 (sec18) transformed with the SUC2 plasmid and SEY2102 transformed with mutant plasmids were derepressed for invertase expression and labeled with $^{35}$SO$_4^{2-}$ for 30 min (37°C for SEY5188 and 25°C for SEY2102). A portion of the SEY2102 culture was incubated an additional 2 h under conditions of chase. Spheroplasts formed from each sample were sedimented, and invertase immunoprecipitated from the pellet (l) and supernatant (E) fractions as before. Samples of each were treated with endo H and compared with untreated material by SDS gel electrophoresis and autoradiography.

### Table II

|          | $^{35}$SO$_4$ in cycle | $[^{3}H]$leu in cycle |
|----------|------------------------|------------------------|
| SUC2     |                        |                        |
| ER       | 2                      | 11                     |
| Secreted | 2                      | ND                     |
| SUC2-s1  |                        |                        |
| ER-unprocessed | 1                   | 2, 3, 7, 9, 10         |
| ER-processed | 1                   | 10                     |
| Secreted | 1                      | ND                     |

met-leu-leu-gln-al-a-phe-leu-phe-leu-ala-gly-phe-ala-ala-lys-ile-

1 2 3 4 5 6 7 8 9 10

|          |          |          |          |
|----------|----------|----------|----------|
| sec18    | wt       | wt       | wt       |
| pRB58    | pRB58-s1 | pRB58-s2 | pRB58-s1 |
| endo H   | −        | +        | −        | +        |

### DISCUSSION

Lesions in a transport signal could, by means of genetic suppressor analysis, lead to the definition of interacting proteins that mediate transport. As a first step in this approach, we have designed a screening procedure to detect mutations in the invertase structural gene that specifically affect secretion. By insisting on retention of full enzyme activity, we should avoid mutations that simply result in denatured protein precipitated in the lumen of a secretory organelle. As an illustration of this point, of 29 independent suc2 mutant isolates that produce enzymatically inactive invertase cross-reacting material, six were found to accumulate in a core-glycosylated form. These mutations most likely yield denatured invertase that remains in the ER.

Starting with extensively mutagenized SUC2 plasmid DNA, only three out of 6,000 transformants were found that accumulate active, stable invertase. Of these, two contain the same mutation. The distinct mutations, SUC2-s1 and -s2, produce fully active forms of invertase which are delayed in transport from the ER (or possibly some post-ER compartment) to the Golgi body, but not to a similar extent in subsequent export to the cell surface. This delay is considerably greater for s1 than s2.

Signal peptide cleavage in the s1 mutant invertase is deficient owing to the substitution of val for ala at position −1 of the cleavage site. Retention of the signal peptide on about half of the s1 molecules causes invertase to form irregular assemblies, as detected by electrophoresis in a nondenaturing gel system. These assemblies remain associated with ER membranes permeabilized by a weak detergent, saponin, while being readily solubilized by Triton X-100. The other half of the accumulated s1 invertase, which has a polypeptide size characteristic of the proteolytically processed form, may be retained by normal subunit association with the unprocessed subunits. Alternatively, as this material is processed at the adjacent peptide bond, met-terminated invertase may be inherently transport defective.

The mutant invertase is cleaved at a reduced rate at the adjacent ser-met bond. This alternate site has, according to von Heijne's rules (49), roughly equal probability of cleavage in the normal invertase signal peptide. Substitution of val at the normal site increases the probability of cleavage at the alternate site. Additional substitutions at the ser site may further reduce processing and secretion of invertase so that cells would be unable to grow on sucrose or raffinose as a carbon source. Such a situation could be used to obtain extragenic suppressor mutations among which signal peptidase mutants with altered substrate specificity may be found.

It is not clear why the failure to remove a signal peptide has such a dramatic effect on invertase transport from the
ER. The reduced solubility and increased heterogeneity of accumulated s1 invertase suggests that unprocessed enzyme either associates with a stable ER component or aggregates to a point where proper packaging for transport is hindered. In either case, the accumulation has no effect on transport of acid phosphatase, or cell surface growth, so it is unlikely that mutant invertase associates with and titrates some essential ER component.

Other similar effects of signal peptide cleavage inhibition have been noted. Haguenauer-Tsapis and Hinnen (50) have deleted the cleavage site region of the yeast acid phosphatase gene (PHO5) and find unprocessed, core-glycosylated enzyme accumulated within the cell which is only slowly secreted. Hortin and Boone (51, 52) report that incorporation of the threonine analogue, β-hydroxyornovaline, at the preprolactin signal cleavage site blocks proper processing. Precursors accumulate in the ER in the unprocessed and in an alternatively processed form. This material is unstable and only slowly secreted in small amounts into the medium. Solubility and membrane association of the unprocessed preprolactin and acid phosphatase have not been investigated; however, failure to cleave the signal peptide of a mutant form of bacterial β-lactamase results in association of the precursor with the external surface of the cytoplasmic membrane (53).

There are many examples of proteins with uncleaved signal peptides, such as yeast α-factor (54), which nevertheless are transported rapidly from the ER. What then is the function of signal peptide cleavage? Wickner (55) has suggested that folding of membrane proteins and perhaps secretory proteins is influenced by the signal peptide. If so, signal peptide cleavage early in the synthesis of a precursor could ensure correct folding. This appears not to be the case for s1 mutant invertase and cleavage-deficient acid phosphatase (50); both are fully active enzymes. Perhaps certain signal peptides are cleaved to prevent association with a stable component of the ER. In this regard, some proteins are designed to remain in the ER, and may retain the signal peptide as a specific anchoring segment.

s2 mutant invertase shows slow transport from the ER with no delay in signal peptide cleavage. With regard to enzyme activity, electrophoretic mobility, ease of release from the ER, and temperature stability, s2 mutant invertase resembles the normal enzyme accumulated in the ER in the pleiotropic mutant sec18. Although the mutation results in a hydrophobic substitution (thr → ile at position +64), no effect on subunit interaction or solubility in the presence or absence of detergent was detected. The effect on transport could be due to a subtle conformational change that influences the protein, or to defective processing. This material is unstable and only slowly secreted from the ER in the unprocessed and in an alternatively processed form. This material is unstable and only slowly secreted from the ER in the unprocessed and in an alternatively processed form. This material is unstable and only slowly secreted from the ER in the unprocessed and in an alternatively processed form.

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