Characterization of New Mutants in the Early Part of the Yeast Secretory Pathway Isolated by a [3H]Mannose Suicide Selection

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Abstract. We have adapted a [3H]mannose suicide selection to identify mutations in additional genes which function in the early part of the yeast secretory pathway. Thus far this protocol has led to the identification of two new genes which are implicated in this process, as well as additional alleles of previously identified genes. The new mutants, bet1 and bet2, are temperature sensitive for growth and protein transport. Thin section analysis has revealed the accumulation of a network of endoplasmic reticulum (ER) at the restrictive temperature (37°C). Precursors of exported proteins that accumulate in the cell at 37°C are terminally core glycosylated. These observations suggest that the transport of precursors is blocked subsequent to translocation into the ER but before entry into the Golgi apparatus. The bet1 and bet2 mutants define two new complementation groups which have the same properties as previously identified ER-accumulating mutants. This and previous findings (Novick, P., C. Field, and R. Schekman, 1980, Cell, 21:205-215) suggest that protein exit from the ER and entry into the Golgi apparatus is a complex process requiring at least 11 genes.

All secreted, lysosomal, and membrane proteins initially use the same transport pathway. The early stages of protein export involve synthesis on cytoplasmic ribosomes, followed by targeting to and exit from the endoplasmic reticulum (ER). Two components involved in the targeting of proteins to the ER have been identified—signal recognition particle and the signal recognition particle receptor, also called the docking protein (for review see Walter et al., 1984). Less is known about subsequent steps. After targeting, secretory proteins must be translocated through the ER membrane; then resident ER proteins must in some way be separated from those to be secreted. Studies demonstrating that different secretory proteins leave the ER at different rates (Fries et al., 1984; Lodish et al., 1983) suggest that these proteins also must be sorted from one another. Two hypotheses have been suggested to explain how this might occur. Blobel (1980) has proposed that protein exit from the ER can occur when specific protein sequences are recognized by their corresponding receptors. It has also been suggested (Kelly, 1985) that proteins move from the ER to the Golgi apparatus by bulk flow, except when specifically restrained or diverted. One such restraining protein, immunoglobulin heavy chain binding protein, has recently been identified (Bole et al., 1986). This binding protein has been shown to bind to immunoglobulin heavy chains and prevent them from leaving the ER until they are complexed to light chains.

To identify other proteins that mediate the interaction of exported proteins with the ER, we are following a genetic approach using the yeast, *Saccharomyces cerevisiae*. This simple eukaryote was shown to have a secretory pathway similar to that of mammalian cells (Novick et al., 1981). Using a technique that enriched for mutants that were denser than wild type at 37°C, Novick et al. (1980) and Ferro-Novick et al. (1984) isolated a collection of yeast mutants that was temperature sensitive (ts) for growth and secretion. The mutants blocked export at four different stages of the yeast transport pathway: entry into the lumen of the ER, exit from the ER, transport out of the Golgi apparatus, and exocytosis. This enrichment required that ts mutants survive a 3-h incubation at 37°C. This incubation was critical to the enrichment, since it was during this time that cells became dense. Therefore, any mutants that would die during such an incubation could not be selected. The density enrichment led to the isolation of a large number of mutants blocked at late stages of the secretory pathway. In contrast, fewer early blocked secretory mutants were identified. Mutations in only two genes, sec53 and sec59, blocked the completion of import of proteins into the ER (Ferro-Novick et al., 1984; Ferro-Novick, 1985). Mutations in nine genes (secl2, secl3, secl6, secl7, secl8, sec20, sec2l, sec22, and sec23) affected transport subsequent to this but before entry into the Golgi apparatus (Novick et al., 1980); these were termed ER-accumulating mutants.

1. Abbreviations used in this paper: CPY, carboxypeptidase Y; endo H, endoglycosidase H; ER, endoplasmic reticulum; ts, temperature sensitive.
based on their EM morphology. The distribution of mutant alleles indicated that more genes were involved in these processes.

It is our goal to identify additional genes that play a role in the early part of the yeast secretory pathway. To develop a protocol that would specifically enrich for mutations affecting these steps, we took advantage of the observation that some yeast glycoproteins contain ~50% carbohydrate (Gascón et al., 1968) that is composed almost entirely of mannose (Ballou, 1976). Most of this carbohydrate appears to be acquired in the Golgi apparatus (Esmon et al., 1981). The rationale behind the technique is that mutants blocked early in the secretory pathway should incorporate less [3H]mannose into their glycoproteins and have a higher probability of surviving the enrichment, while wild-type cells or mutants blocked late in the pathway should incorporate more [3H]mannose and have reduced viability. A [3H]mannose suicide selection has been used by Hufnaker and Robbins (1982, 1983) to isolate glycosylation mutants. It was also found that mutations in the sec53 gene could be isolated using this selection (Hufnaker and Robbins, 1983). We adapted the protocol of Hufnaker and Robbins so it could be used to isolate additional early blocked secretory mutants. The screen we developed to identify such mutants, as well as the characterization of these new mutants, is described.

Materials and Methods

Media and Growth Conditions

YP medium contained 1% Bacto-Yeast Extract and 2% Bacto-Peptone; YPD medium was the same with 2% glucose. Phosphate-depleted YPD was as described by Rubin (1973). Wickerham's minimal medium (Wickerham, 1946) was supplemented with either histidine (20 µg/ml) or uracil (40 µg/ml) and used with varying amounts of glucose; for sulfate-free medium, chloride salts replaced all sulfate salts. To change growth medium, cells were sedimented in a clinical centrifuge at room temperature and then resuspended in fresh medium. Cell density was measured in a 1-cm quartz cuvette at 599 in a model No. 25 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA); 1 OD599 U corresponds to 0.15 mg dry weight.

Reagents and Buffers

Reagents were obtained as indicated: glucose oxidase was from Boehringer Mannheim Biochemicals (Indianapolis, IN); zymolyase 100T and enolase from Sigma Chemical Co. (St. Louis, MO); Spurr embedding medium was a gift from Dieter Wolf. Antibody to invertase was prepared as described. An [3H]mannose suicide selection has been done by immunoprecipitation using the protocol described below and new sec53 alleles were identified by complementation analysis. Antibody to CPY was used to screen potential secretory mutants for a failure to process this vacuolar protein. This was done by immunoprecipitation using the protocol described below.

Immunoprecipitation and Electrophoresis

Cells were grown to exponential phase in minimal medium supplemented with 100 µM ammonium sulfate and 2% glucose. The protocol used differed somewhat depending on the protein immunoprecipitated. In general, aliquots (1 OD599 U) were incubated at 37°C for 30 min, pelleted, and resuspended in 0.5 ml of minimal medium supplemented with 20-25 µM ammonium sulfate, 2% glucose (or 0.1% glucose to derepress the synthesis of invertase), and 200 µCi of [35S]sulfate. The incubation was then continued for an additional 30-45 min. At the end of the incubation, the cells were washed once with 1 ml of cold 10 mM sodium azide and resuspended in 0.5 ml of the same. To convert cells to spheroplasts, 2× spheroplast medium containing 1 U of zymolysate was added and the samples were incubated at 30°C for 1 h. Samples were centrifuged in a clinical centrifuge for 5 min; the spheroplast pellet was lysed in 1% SDS and heated for 4 min in a boiling water bath. Lysates were diluted with 0.9 ml of dilution buffer and centrifuged in an Eppendorf centrifuge for 15 min. The supernatant (0.8 ml) was removed and incubated with one of three antibodies (2 µl of anti-invertase antibody; 1 µl of anti-CPY antibody; 2 µl of anti-a-factor antibody) at 0°C for 16 h. Protein A-Sepharose beads (60 µl of a 10% solution) were added and samples were incubated with shaking for 30-90 min at 4°C. The beads were washed four times with 1.0 ml of wash buffer and then once with 1 ml of cold PBS. For immunoprecipitations with anti-CPY antibody, beads were washed as described by Stevens et al. (1982). To solubilize antigen-antibody complexes, the Protein A-Sepharose was resuspended in Laemmli sample buffer and heated in a boiling water bath for 3 min. When samples were treated with endo H, the beads were resuspended in endo H buffer and heated to 100°C for 5 min. Samples were diluted with 0.1 M sodium acetate (pH 5.6) buffer containing protease inhibitors and incubated with endo H for 16 h at 37°C. The final mixture contained 0.1% SDS, 80 U/ml endo H, and 1 µg/ml of leupeptin, chymostatin, pepstatin, antipain, and aprotinin. Laemmli buffer (3×) was added and samples were heated to 100°C for 3 min before they were subjected to SDS-PAGE.

All samples were subjected to electrophoresis using 80 (or 15%) SDS-PAGE (Laemmli, 1970). To compare strains, the volume of sample loaded was directly proportional to the incorporation of [35S]sulfate in the lysate. Molecular mass markers were as follows: myosin, 205 kDa; β-galactosidase, 116 kDa; phospholipase B, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa. Gels were stained for protein, destained, washed twice with 10% methanol (15 min each time), and prepared for fluorography by treatment with 1.0 M sodium salicylate in 10% methanol as described before (Chamberlain, 1979). Autoradiography was performed using preflashed Kodak XAR-5 film.

Enzyme Assays and Other Procedures

External invertase was assayed as described by Goldstein and Lampen (1975); units of activity are µmoles of glucose released per minute per OD599 U of cells. Internal invertase was assayed in spheroplast lysates. Cells (1 OD599 U) were converted to spheroplasts during an incubation (30°C for 45 min) in spheroplast medium containing 5 U of zymolysate. Spheroplasts were pelleted and lysed with 0.5 ml of cold 0.5% Triton X-100; 10 µl of the lysate was assayed at 37°C for 15 min using the protocol of Gold-
Table I. Strains Used in This Study

| Strain          | Genotype   | Source                  |
|-----------------|------------|-------------------------|
| SFNY26-3A       | MATα, ura3-52 | This study              |
| SFNY26-5A       | MATα, ura3-52, sec59-1 | This study              |
| SFNY26-5B       | MATα, his4-619 | This study              |
| NY13            | MATα, ura3-52 | P. Novick*              |
| NY417           | MATα, ura3-52, sec17-1 | P. Novick              |
| NY432           | MATα, ura3-52, sec18-1 | P. Novick              |
| ANY113*         | MATα, ura3-52, his4-619, bet1-1 | This study            |
| ANY119*         | MATα, ura3-52, his4-619, bet2-1 | This study            |

* SFNY26-3A and SFNY26-5B were constructed by crossing X2180-1A (from the Yeast Genetics Stock Center) to DBY877 (obtained from D. Botstein, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA) seven times.

† ANY113 and ANY119 were obtained by crossing original isolates (E-336 and E-383) to SFNY26-3A.

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Results

Mutant Isolation and Screen

To isolate new ts mutants that block transport early in the secretory pathway, strain SFNY 26-5B (1.9 × 10⁶ cells) was subjected to mutagenesis with ethylmethanesulfonate and then incubated with [3H]mannose at 37°C. After 2 mo at ~80°C, 1.67 × 10⁶ cells survived (see Table II), indicating that two logs of killing had been obtained. Of the survivors, 406 colonies were found to be temperature sensitive for growth. To identify those mutants that displayed a temperature-dependent block in protein secretion, strains were first assayed for their ability to secrete the yeast glycoprotein invertase. It was found that 63 of the ts mutants secreted a significantly smaller portion of wild-type invertase at 37°C (restrictive temperature) than at 24°C (permissive temperature). Of the 63 mutants, five were found to be defective in protein synthesis at the restrictive temperature; the remaining mutants were all determined to be recessive by complementation analysis.

Since the [3H]mannose suicide selection had previously resulted in the isolation of the sec53 (alg4) mutant (Huffaker and Robbins, 1983), it seemed likely that some of the mutants isolated in this study might be alleles of this gene. Complementation analysis revealed that three of these mutants were new alleles of the sec53 gene.

When secretion is blocked, the precursors that accumulate inside the cell are indicative of the stage at which transport is arrested (Esmon et al., 1981; Ferro-Novick et al., 1984). To screen the remaining mutants, we chose to follow the transport of the yeast vacuolar protease CPY. CPY is synthesized as a 59-kD polypeptide to which 10 kD of N-glycosidically linked carbohydrate is added (Hasilik and Tanner, 1978). Proteolytic activation of proCPY takes place in the vacuole and involves cleavage of an 8-kD propeptide by the PEP4 gene product (Hemnings et al., 1981; Ammerer et al., 1986; Woolford et al., 1986) to yield a mature CPY molecule of 61 kD. Transport of the proenzyme requires the early part of the secretory pathway (Stevens et al., 1982; Ferro-Novick et al., 1984). Yeast sec mutants that block transport at or before the Golgi apparatus fail to transport CPY to the vacuole. Mutants which disrupt entry into the lumen of the ER synthesize predominantly a 59-kD unglycosylated form of proCPY (Ferro-Novick et al., 1984). Other mutants synthesize either a 67-kD (pl CPY) incompletely glycosylated form (ER-accumulating mutants) or a fully glycosylated 69-kD (p2 CPY; Golgi-accumulating mutants) form (Stevens et al., 1982).

Mutants to be screened were incubated at the restrictive temperature and radiolabeled with [35S]sulfate. Cell lysates were prepared and immunoprecipitated with anti-CPY antibody. Many of the mutants that were screened did not effect the processing of CPY. Two mutants (E-336 and E-383), however, synthesized the pl form of CPY (Stevens et al., 1982) that is also synthesized in the sec18 mutant at 37°C (Fig. 1, lanes 1–3). The sec18 mutant is a previously identified ER-accumulating mutant that blocks transport from the ER to the Golgi complex (Novick et al., 1980). To determine whether the CPY protein moiety synthesized in the sec18, E-336, and E-383 mutants is the same, endo H digestion was performed on radiolabeled immunoprecipitated CPY. Endo H cleaves between the two N-acetylglucosamine units that connect N-linked oligosaccharides to protein, removing all carbohydrate from the protein, except for one N-acetylglucosamine unit. After endo H digestion, the electrophoretic mobility of the CPY synthesized in sec18 was the same as that synthesized in the E-336 and E-383 mutants (data not shown).

To determine whether the observed phenotype was due to a single or multiple ts mutation(s), E-336 and E-383 were

Table II. Data from [3H]Mannose Suicide Selection

| Cells stored at ~80°C | 1.9 × 10⁶ |
|----------------------|----------|
| Cells surviving suicide | 1.67 × 10⁶ |
| Mutants ts for growth | 406 |
| Mutants ts for invertase secretion | 63 |
| Mutants defective in protein synthesis | 5 |
| sec53 mutants | 3 |
| Additional mutants synthesizing early forms (CPY) | 2 |

Note that if cells are stored at ~80°C for a longer period of time, the number of survivors diminishes. When 2.7 × 10⁶ cells survive the enrichment, the number of sec53 mutants found among the survivors is the same.
Figure 1. Immunoactive CPY synthesized at 37°C. Cells were shifted to 37°C for 30 min in minimal medium containing 20 μM ammonium sulfate plus 2% glucose and then labeled with 200 μCi of [35S]sulfate at 37°C for 30 min in the same medium. Radiolabeled cells were converted to spheroplasts and lysed as described in the Material and Methods. CPY was immunoprecipitated and analyzed by (10%) SDS-PAGE: (lane 1) E-336 (ANY113); (lane 3) E-383 (ANY119); (lane 4) wild-type SFNY26-3B.

crossed to wild type, sporulated, and the resulting diploids were analyzed by tetrad analysis. In the case of E-336, 10 tetrads were analyzed; the ts growth defect showed 2:2 segregation which always coincided with the secretion defect. For E-383, 11 tetrads were analyzed; the growth defect showed 2:2 segregation, but the secretion defect was too leaky to substantiate this proposal, the secretion of acid phosphatase was measured. Acid phosphatase (see Table III) is a periplasmic enzyme in yeast whose synthesis is derepressed in the absence of phosphate. Mutants and wild type were either derepressed for 2.5 h at 24°C or, partially derepressed for 2.5 h at 24°C and then shifted to 37°C for 2.5 h longer. The amount of external acid

Table IV. Reversibility of Accumulated Invertase (U/mg dry weight)

| Strain     | Description | 1 h (37°C) | 3 h (24°C) | 24°C | 1 h (37°C) | 3 h (37°C) | 37°C | 1 h (24°C) |
|------------|-------------|------------|-----------|------|------------|------------|------|------------|
|            |             | External   | Internal  | External | Release | External | Release | External   |
| SFNY26-3A  | Wild type   | 0.34       | 0.14      |        |          |           |      |            |
| NY 432     | sec18-1     | 0.01       | 0.65*     | 0.43  | 65        | 0.04      | 5    | 0.40       |
| ANY113     | bet1-1      | 0.06       | 0.43*     | 0.33  | 63        | 0.15      | 21   | 0.34       |
| ANY119     | bet2-1      | 0.18*      | 0.32      | 0.32  | 44        | 0.26      | 25   | 0.34       |

Cells were grown overnight at 24°C to early exponential phase in YPD medium supplemented with 7 mM potassium phosphate. Cells (4.5 OD600 U) were pelleted, resuspended in 3.0 ml of phosphate-depleted YPD medium (Rubin, 1973), and incubated at 24°C for 2.5 h. Cells were then divided into three aliquots: 1.0 ml was transferred to 37°C, 1.0 ml was left at 24°C, and the remaining portion was placed on ice. Samples at 24 or 37°C were incubated for an additional 2.5 h. At the end of the incubation, cells were washed with 10 mM sodium azide, resuspended in 1.0 ml of sodium azide, and assayed.

Table III. Acid Phosphatase Secretion

| Strain     | Description | 2.5 h (24°C) | 2.5 h (37°C) | 2.5 h (24°C) |
|------------|-------------|-------------|-------------|-------------|
| SFNY26-3A  | Wild type   | 19          | 108         | 100         |
| NY 432     | sec18-1     | 29          | 36          | 138         |
| ANY113     | bet1-1      | 16          | 19          | 96          |
| ANY119     | bet2-1      | 11          | 38          | 68          |

Cells were grown overnight at 24°C to early exponential phase in YPD medium supplemented with 7 mM potassium phosphate. Cells (4.5 OD600 U) were pelleted, resuspended in 3.0 ml of phosphate-depleted YPD medium (Rubin, 1973), and incubated at 24°C for 2.5 h. Cells were then divided into three aliquots: 1.0 ml was transferred to 37°C, 1.0 ml was left at 24°C, and the remaining portion was placed on ice. Samples at 24 or 37°C were incubated for an additional 2.5 h. At the end of the incubation, cells were washed with 10 mM sodium azide, resuspended in 1.0 ml of sodium azide, and assayed.

Table I. Reversibility of Accumulated Invertase (U/mg dry weight)

| Strain     | Description | 1 h (37°C) | 3 h (24°C) | 24°C | 1 h (37°C) | 3 h (37°C) | 37°C | 1 h (24°C) |
|------------|-------------|------------|-----------|------|------------|------------|------|------------|
|            |             | External   | Internal  | External | Release | External | Release | External   |
| SFNY26-3A  | Wild type   | 0.34       | 0.14      |        |          |           |      |            |
| NY 432     | sec18-1     | 0.01       | 0.65*     | 0.43  | 65        | 0.04      | 5    | 0.40       |
| ANY113     | bet1-1      | 0.06       | 0.43*     | 0.33  | 63        | 0.15      | 21   | 0.34       |
| ANY119     | bet2-1      | 0.18*      | 0.32      | 0.32  | 44        | 0.26      | 25   | 0.34       |

Cells were grown overnight at 24°C to early exponential phase. Samples (3.0 OD600 U) were incubated at 37°C for 30 min, pelleted, resuspended in 3.0 ml of prewarmed YP medium containing 0.1% glucose, and incubated at 37°C for an additional 60 min. Aliquots (1.0 ml) were pelleted, resuspended in YPD medium containing 0.1 mg/ml cycloheximide, and incubated at 24 or 37°C for 3 h; the remaining cells were placed at 0°C. At the end of all incubations, cells were washed with 10 mM sodium azide and resuspended in 1.0 ml of cold sodium azide. The percent release at 24°C was calculated as (4 h external [37°C–24°C] – 1 h external invertase [37°C]/1 h internal invertase [37°C]). Parallel samples were incubated at 24°C for 1 h.

The Mutants Have Pleiotropic Defects in Secretion

The new secretory mutants, bet1-1 and bet2-1, were identified as being temperature sensitive for invertase secretion (see above; data quantitated in Table IV) and defective in the processing of the vacuolar protease CPY. These findings suggest that the transport defect in bet1 and bet2 is pleiotropic. To substantiate this proposal, the secretion of acid phosphatase was measured. Acid phosphatase (see Table III) is a periplasmic enzyme in yeast whose synthesis is derepressed in the absence of phosphate. Mutants and wild type were either derepressed for the synthesis of acid phosphatase for 5 h at 24°C, or, partially derepressed for 2.5 h at 24°C and then shifted to 37°C for 2.5 h longer. The amount of external acid

If cells were not preincubated at 37°C, before the derepression of invertase, 1.049 and 0.647 U/mg dry weight of invertase was found to accumulate within the sec18 and bet1 mutants, respectively. The bet2 mutant secreted 0.295 U/mg dry weight of external invertase if cells were not preincubated before the derepression of invertase.
phosphatase secretion was either essentially the same as in wild type at 37°C (see Table III). However, at 24°C, acid phosphatase secretion was either essentially the same as in wild type (\( \text{bet1} \)) or somewhat lower (\( \text{bet2} \)). These findings suggest that the transport defects in the \( \text{bet1} \) and \( \text{bet2} \) strains are pleiotropic.

Precursor Proteins Accumulate Inside the Cell

A failure to export proteins to the cell surface may result in an accumulation of protein precursors within the cell. To determine whether there is an intracellular accumulation of invertase in \( \text{bet1} \) and \( \text{bet2} \), cells were first labeled with \( [\text{35S}] \) sulfate at 37°C, during the derepression of invertase synthesis. The yeast cell wall was then enzymatically removed to generate spheroplasts and the spheroplasts were spun to separate the contents retained in the cell from those released into the periplasm. Spheroplasts were then lysed and immunoprecipitated with anti-invertase antibody. As shown in Fig. 2 (lanes 1–3), the \( \text{sec18, bet1,} \) and \( \text{bet2} \) mutants accumulate a stair ladder of invertase bands of 79, 81, and 83 kD that are not seen in wild-type (lane 4). These forms have been previously identified in ER-accumulating mutants and shown by several criteria to be indicative of protein core glycosylation only (Esmon et al., 1981). Therefore, the precursors in these mutants must be accessible to the glycosylating machinery of the ER, but not to the glycosyltransferases of the Golgi apparatus.

The transport of the secreted yeast pheromone \( \alpha \)-factor in the \( \text{bet1 and bet2} \) mutants was also examined. Yeast \( \alpha \)-factor is synthesized as an 18.6-kD precursor (Kurjan and Herskowitz, 1982; Emter et al., 1983; Julius et al., 1984). Three core oligosaccharides are added in the lumen of the ER, yielding a 26-kD polypeptide (Emter et al., 1983; Emter et al., 1984). Mutants were radiolabeled at the restrictive temperature and immunoprecipitated with anti-\( \alpha \)-factor antibody. As controls, \( \text{sec59, sec17, sec18,} \) and wild type were also radiolabeled and immunoprecipitated with \( \alpha \)-factor antibody. (Fig. 3 shows that the major form of \( \alpha \)-factor accumulating in \( \text{bet1 and bet2} \) (lanes 5 and 6) has the same molecular mass (26 kD), as found in two different ER-accumulating mutants, \( \text{sec17 and sec18} \) (lanes 3 and 4). The predominant form of \( \alpha \)-factor accumulating at 37°C in \( \text{sec59,} \) a mutant which disrupts entry into the ER, is the 18.6-kD species (Fig. 3, lane 1). It should be noted that minor intermediate forms of \( \alpha \)-factor can be detected in all of the mutants (see Fig. 3). No accumulated \( \alpha \)-factor was observed in wild type (lane 2). The enrichment previously used to isolate secretory mutants in yeast required that cells become dense at the restrictive temperature (Novick et al., 1980; Ferro-Novick et al., 1984). It was hypothesized that the \( \text{sec} \) mutants become dense because net cell surface growth is stopped while cell mass continues to increase. Interestingly, both \( \text{bet1 and bet2} \) become as dense as \( \text{sec18} \) following a shift to the restrictive temperature (data not shown), suggesting that this may be a general property of mutants blocked at this stage of transport.

The Accumulated Invertase Is Enzymatically Active

Yeast cells synthesize two forms of invertase: a constitutively synthesized cytoplasmic form and a secreted form whose synthesis is under hexose repression (Perlman and Halvorson, 1981; Carlson and Botstein, 1982). The early mutants

Figure 2. Immunoreactive invertase accumulated within \( \text{bet1 and bet2} \) at 37°C. Cells were shifted to 37°C for 30 min in minimal medium containing 100 \( \mu \text{M} \) ammonium sulfate plus 2% glucose. At the end of the incubation, cells were pelleted, resuspended in minimal medium containing 25 \( \mu \text{M} \) ammonium sulfate plus 0.1% glucose, and radiolabeled with 200 \( \mu \text{Ci} \) of \( [\text{35S}] \) sulfate at 37°C for 30 min. Radiolabeled cells were converted to spheroplasts and lysed as described in the Material and Methods. Invertase was immunoprecipitated and analyzed by (10%) SDS-PAGE: (lane 1) \( \text{sec18} \) (NY432); (lane 2) \( \text{bet1} \) (NY113); (lane 3) \( \text{bet2} \) (NY113); (lane 4) wild type (SFNY26-3A).

Figure 3. Immunoreactive \( \alpha \)-factor accumulated in \( \text{bet1 and bet2} \) at 37°C. Cells were shifted to 37°C for 30 min in minimal medium containing 25 \( \mu \text{M} \) ammonium sulfate plus 2% glucose and then labeled with 200 \( \mu \text{Ci} \) of \( [\text{35S}] \) sulfate at 37°C for 45 min in the same medium. Radiolabeled cells were converted to spheroplasts and lysed as described in the Materials and Methods. \( \alpha \)-Factor was immunoprecipitated and analyzed by (15%) SDS-PAGE (lane 1) \( \text{sec59} \) (SFNY26-5A); (lane 2) wild-type (SFNY26-5B); (lane 3) \( \text{sec17} \) (NY417); (lane 4) \( \text{sec59} \) (NY432); (lane 5) \( \text{bet1} \) (NY113); (lane 6) \( \text{bet2} \) (NY119).
sec53 and sec59 accumulate enzymatically inactive forms of invertase (Ferro-Novick et al., 1984), whereas all previously identified ER-accumulating mutants contain enzymatically active invertase internally (Novick et al., 1980). When the bet1 and bet2 mutants are incubated at the restrictive temperature, a precursor form of invertase also accumulates inside the cell (Fig. 2, lanes 2 and 3). The bet1 and bet2 strains were therefore assayed to see if the invertase they accumulate is enzymatically active. Mutants were shifted to 37°C for 30 min and then resuspended in medium containing 0.1% glucose to derepress the synthesis of invertase. Cells were converted to spheroplasts and pelleted. The cell pellet was lysed and assayed for invertase activity. As can be seen in Table IV, both bet1 and bet2 accumulate enzymatically active invertase; the activity detected in wild type is largely due to cytoplasmic invertase. If sec18 or bet1 is not preincubated at 37°C, before the derepression of this enzyme, more invertase accumulates inside the cell (see legend to Table IV). The bet2 mutant, however, was found to display a stronger block in invertase secretion after a preincubation at 37°C (Table IV).

To determine the extent to which the invertase that accumulates inside bet1 and bet2 can be reversibly secreted, the following experiment was done. Cells were allowed to accumulate active invertase at 37°C, and were then shifted to 24°C in the presence of cycloheximide to inhibit further protein synthesis. To control for the leakage of accumulated invertase that would occur during a prolonged incubation, a parallel experiment at 37°C was also done. The data in Table IV indicates that both mutants showed an increase in the level of external invertase secreted into the cell wall after a shift down to 24°C. For the bet1 mutant, this increase is significantly greater after an incubation at 24°C, as compared to 37°C. Thus, the secretion defect is partially reversible. In the bet2 mutant, the amount that is secreted after equivalent incubations at 37 and 24°C is not dramatically different. Yet this mutant is still somewhat reversible.

The Mutants Accumulate ER Membrane at the Restrictive Temperature

Since previously identified sec mutants have been shown to accumulate secretory organelles at the restrictive temperature (Novick et al., 1980), thin sections of wild type, bet1 and bet2 mutant cells were examined by electron microscopy. In wild type, thin tubules of ER are often seen in contact with the nuclear membrane or at the cell's periphery. When bet1 and bet2 are incubated at 37°C for 2 h, several distinctive features can be seen in both mutants. The mutants accumulate more ER membrane than seen in wild type (Fig. 4; compare A, B, and C). The lumen of the ER and nuclear envelope is considerably dilated when compared to wild type and patches of small vesicles can be seen in the cytoplasm; the nucleus is also multi-lobed. The morphology of bet1 and bet2 is similar to that seen in members of three of the nine previously identified complementation groups of ER-accumulating sec mutants (Novick et al., 1980); members of the other six complementation groups accumulated ER, but no small vesicles. The vesicles observed in bet1 (30-nm average diameter) are comparable in size to those seen in the ER-accumulating sec mutants (40 nm). The bet2 mutant also contains vesicles of this size. In addition to these 30-nm vesicles, the bet2 mutant contains 80-100-nm vesicles like those seen in the exocytosis mutants (Novick et al., 1980). This is Figure 4. Electron micrographs of cells incubated at 37°C for 2 h. (A) bet1-1 (ANY 113); (B) bet2-1 (ANY 119); (C) wild-type (NY 13). er, endoplasmic reticulum; n, nucleus; np, nuclear pore; sv, small vesicles. Bar, 1 μm.
compatible with the observation that the block in secretion
is not tight in bet2 (see Tables III and IV) and some transport
beyond the initial block can occur. Previous studies with
double mutants suggest that the small vesicles seen in some
of the ER-accumulating mutants are not vesicular carriers
mediating transport from the ER to the Golgi complex (Nov-
wick et al., 1981).

Discussion

A [3H]mannose suicide selection has been used to isolate
mutants that define two new genes (bet1 and bet2) which ap-
pear to function in the early part of the yeast secretory path-
way. The mutants bet1 and bet2 accumulate ER membrane
when incubated at the restrictive temperature. These mutants
block the transport of invertase, acid phosphatase, and α-fac-
tor to the cell surface, and fail to complete processing of the
vacular protease CPY. The pleiotropy of the block suggests that
the affected genes code for proteins which play a role in
the secretory pathway. The extent of processing of glycopro-
teins in the mutants is indicative of precursors that are acces-
sible to the glycosylating enzymes of the ER, but not to the
glycosyltransferases of the Golgi apparatus. The effects on
protein transport are partially reversible; when the mutants
are shifted back down to the permissive temperature, in the
presence of cycloheximide, some of the accumulated inver-
tase is reversibly secreted. Mutations in the bet1 and bet2
genes define two new complementation groups of ER-accum-
lulating mutants. Like previously identified ER-accumu-
lating mutants, these new mutants also become dense at the re-
strictive temperature. Thus, an increase in cell density with
the onset of a block in transport appears to be a general prop-
erty of these mutants. Mutants with defects in other genes
have also been isolated as part of the [3H]mannose suicide
selection. For example, additional alleles of the sec53 gene,
a previously identified mutant that disrupts entry into the
lumen of the ER (Ferro-Novick et al., 1984) have been ob-
tained (Huffaker and Robbins, 1983; this study). Therefore,
the [3H]mannose suicide selection should also be useful in
identifying mutations in new genes that can disrupt translo-
cation.

It is likely that further genetic studies will implicate more
genesis in the process of protein export from the ER. This is
suggested by the fact that two new ER-accumulating muta-
tions were identified in this study, while no new alleles of pre-
viously identified ER-accumulating mutants were found. Thus,
at least eleven, and probably more, genes are involved in the
processes of protein exit from the ER and arrival to the Golgi
complex. The fact that almost all of these mutants act quickly
suggests a direct role for the corresponding gene products.
The ER-accumulating mutants will allow us to clone wild-
type copies of the affected genes by complementation, and
eventually antibodies to the gene products can be obtained.
The antibodies will make it possible to localize the gene
products in wild-type cells. Localization of the many pro-
teins that appear to function in this stage of protein secretion
will be an important step towards gaining a complete under-
standing of this complex process. This should pave the way
for a more detailed biochemical analysis of the early stages of
protein transport in yeast.

We thank Tim Huffaker for advice on the [3H]mannose protocol; Phil
Robbins for his generous gift of [3H]mannose; Elizabeth Jones, Joel Rothman,
and Tom Stevens for CPY antibody; David Botstein for α-factor antibody;
David Botstein and Peter Novick for strains. We thank Hannelle Ruohola
for helpful discussions throughout the work. We also thank Peter Novick
and Hans Stukenbrok for their expert assistance in the thin section analysis
of the mutants; Ann Curley-Whitehouse for assistance in photography; and
Anthony Lamantia for helpful advice and assistance in determination of
vesicle sizes.

This work was supported by a National Institutes of Health grant (GM
35421-02) and by grant RR 05358 awarded to S. Ferro-Novick by the Bio-
chemical Research Support Grant Program, Division of Research Re-
sources, National Institutes of Health. A. Newman is a recipient of a Na-
tional Science Foundation predoctoral fellowship, and S. Ferro-Novick is
a recipient of a Swedilus Cancer Research Award.

Received for publication 13 April 1987, and in revised form 23 June 1987.

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