Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae

(secretory mutants/vesicles/membrane assembly)

PETER NOVICK AND RANDY SCHEKMAN

Department of Biochemistry, University of California, Berkeley, California 94720

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ABSTRACT Saccharomyces cerevisiae cells contain a small internal pool of the secretory enzymes invertase and acid phosphatase. This pool increases up to 8-fold at 37°C in a temperature-sensitive, secretion-defective mutant strain (sec 1-1). Cell division and incorporation of a sulfate permease activity stop abruptly at the restrictive temperature, while protein synthesis continues for several hours. Electron microscopy of mutant cells incubated at 37°C reveals a large increase in the number of intracellular membrane-bound vesicles, which are shown by histochemical staining to contain the accumulated acid phosphatase. The vesicles are removed and the accumulated enzymes are secreted when cells are returned to a permissive temperature in the presence or absence of cycloheximide. These results are consistent with a vesicle intermediate in the yeast secretory pathway and suggest that exocytosis may contribute to cell-surface growth.

Protein secretion by plant and animal cells is mediated by a complex, highly organized series of membrane-bound structures (1, 2). The mechanism of glycoprotein secretion in Saccharomyces cerevisiae is less well understood. The tight coupling of protein synthesis with secretion and the low frequency of recognizable structures clearly associated with this process have prevented the formation of a coherent model. Membrane-bound vesicles have been implicated in the secretion of β-glucanases (3) and in bud (4) and division septum assembly (5); however, the role of vesicles in the secretion of acid phosphatase (6) and invertase (7) and in plasmalemma assembly has been less clear.

We have developed a genetic approach to the study of the secretory process in yeast. The analysis of a strain with a conditional, reversible block in the secretory pathway has allowed the identification of a vesicular intermediate in secretion and cell-surface growth.

MATERIALS AND METHODS

Materials. S. cerevisiae haploid strain X2180-1A was from the yeast genetics stock center. A constitutive high acid phosphatase-producing strain A137 (α, ACP 1-2, pho 80) and an acid phosphatase-defective strain Ela (a, acp 1) were obtained from P. Hansche (University of California, Davis; ref. 8). HMSF-1 (a, sec 1-1) was derived from X2180-1A. Standard genetic techniques were used to construct SF 150-5c (a, ACP 1-2, pho 80, sec 1-1), and SF 154-10a (a, acp 1, sec 1-1).

YPD medium contained 1% Bacto-Yeast Extract, 2% Bacto-Peptone, and 2% glucose. Wickerham’s minimal medium (9) was used with the following modifications: for phosphate-free medium, potassium chloride replaced potassium phosphate; for sulfate-free medium, chloride salts replaced all sulfate salts. Unless otherwise indicated, the carbon source was 2% glucose. Petri plates contained minimal medium and 2% Difco agar. Liquid cultures were grown in flasks with agitation, and the experiments were initiated with exponentially growing cells at an A600 of 1.5–2.5. When a change in the growth medium was required, the cells were collected by centrifugation, washed twice with distilled water, and resuspended in the new medium. The absorbance of cell suspensions was measured in a 1-cm quartz cuvette at 600 nm in a Zeiss PMQ II spectrophotometer; 1 A600 unit corresponds to 0.15 mg dry weight under all conditions of growth tested. Cell number was determined with a hemocytometer; buds were counted as cells.

Other reagents were obtained as indicated: Ethyl methane-sulfonate, p-nitrophenylphosphate, glucose oxidase, dianisidine, peroxidase, cycloheximide, and homocysteine thiolactone-HCl were from Sigma; H235SO4, L-[4,5-3H]leucine, L-leucine, and L-methionine were from ICN; mycostatin (nystatin) was from Calbiochem; gluuslate was from Endo Laboratories (Garden City, N. J.). Lyticase is a yeast lytic enzyme preparation (unpublished observations), useful in spheroplast formation (10). Fraction II (30,000 units/mg; 1 unit will lyse 0.2 A600 of logarithmic phase S. cerevisiae in 30 min at 30°C) was used.

Isolation of Secretory (sec) Mutants. X2180-1A cells were grown in YPD medium and treated with 3% ethyl methanesulfonate for 60 min at 25°C; the survival rate was 50–70%. The mutagenized culture was diluted with an equal volume of 12% sodium thiosulfate, and the cells were centrifuged and washed twice with distilled water. The cells were then grown in YPD medium for 8 hr at 24°C, and diluted aliquots were spread on minimal medium agar plates. After 3 days at 22–24°C, 1600 colonies were replica-plated onto YPD medium and incubated overnight at 37°C. The temperature-sensitive clones (87/1600) were replica-plated onto phosphate-free minimal medium to derepress the synthesis of acid phosphatase, and after 10 hr at 24° or 37°C the replicas were stained for secreted acid phosphatase (8). The clones that showed temperature-sensitive secretion of phosphatase were screened for conditional secretion of invertase. Cultures grown at 24°C in liquid minimal medium containing 5% glucose were shifted to fresh medium containing 2% sucrose, and after 5 hr at 24° or 37°C the cells were centrifuged, washed with distilled water, and assayed for secreted invertase. Two clones showed conditional secretion but normal incorporation of 35SO4 into protein at 37°C (data not shown). The mutant loci designated sec 1-1 and sec 2-1 are nonallelic and recessive. Only sec 1-1 will be described in this report.

Assays. External (cell wall-bound) invertase was assayed at 37°C as described by Goldstein and Lampen (11); units of activity are μmol of glucose released per min. External acid phosphatase was assayed at 37°C as described by van Rijn et al. (12); units of activity are nmol of p-nitrophenol released per min. Sulfate permease activity was assayed at 37°C in 50 μM

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(NH₄)₂SO₄ as described by Breton and Surdin-Kerjan (13); units of activity are nmol of SO₄²⁻-uptake per min. Internal acid phosphatase and invertase were assayed in spheroplast lysates. Washed cells (10 A₆₀₀ units) were resuspended in 2 ml of 1.4 M sorbitol/0.1 M potassium phosphate, pH 7.5/0.5 mM sodium azide/20 mM 2-mercaptoethanol/80 units of lyticase. After 45 min at 37°C the spheroplasts were centrifuged at 10,000 × g for 5 min, and the pellets were resuspended in 0.5 ml of 1% Triton X-100. Residual 2-mercaptoethanol present in the spheroplast lysate was eliminated with N-ethylmaleimide (0.4 mM), which was added after the first stage of the internal invertase assay. Protein synthesis was measured in 1-ml aliquots of minimal medium containing 1 A₆₀₀ unit of cells and 50 nCi of L-[³H]leucine (0.33 Ci/mol, 1 Ci = 3.70 × 10¹⁰ Bq). Incorporation was stopped after 10 min with 1 ml of cold trichloroacetic acid (20%). After 1 hr at 0°C the mixtures were filtered on Whatman GF/A filters; the filters were washed and dried, and the radioactivity was measured in a Searle Delta 300 liquid scintillation counter.

Electron Microscopy. The procedure of Byers and Goetsch (14) was used for preparation of samples, except for the initial glutaraldehyde fixation, which was done at 0°C. A modification of the procedure of van Rijn et al. (15) was used for the histochemical localization of acid phosphatase activity. Spheroplasts, prepared from 200 A₆₀₀ units of cells and 8000 units of lyticase by the procedure described above, were sedimented and resuspended in 13 ml of 1.4 M sorbitol/0.1 M sodium cacodylate, pH 6.0/5 mM CaCl₂/3% glutaraldehyde and incubated at 0°C for 1 hr. The fixed spheroplasts were centrifuged, washed once with 50 mM sodium acetate (pH 5.5), and resuspended in 16 ml of acetate buffer containing 8.2 mM p-nitrophenylphosphate, 2.3 mM lead nitrate, 2.5% dimethyl sulfoxide, and 0.5 mg of mycogenin per ml. After 1 hr at 30°C the stained spheroplasts were processed as above (14), with the exception that the uranyl acetate and post-staining treatments were eliminated.

RESULTS

Thermoreversible Accumulation of Secretory Proteins. HMSF 1 showed temperature-sensitive growth and secretion of acid phosphatase and invertase as determined by the screening procedure described in Materials and Methods. Synthesis of acid phosphatase was followed in liquid cultures of wild-type (X2180-1A) and HMSF 1 at 24°C and 37°C (Fig. 1). Secreted phosphatase activity was detected in whole cells after 1.5 hr of growth in phosphate-free minimal medium at 24°C. Phosphatase secretion continued at 37°C in X2180; however, in HMSF 1 secretion was blocked, and the previous cell-wall phosphatase activity decayed. Although the intracellular phosphatase level in X2180 cells (measured in extracts of spheroplasts, Fig. 1B) did not vary significantly, the pool in mutant cells increased 5-fold during a 2.5-hr incubation at 37°C. The intracellular phosphatase activity decreased during a subsequent 2.5-hr incubation at 24°C with the presence of cycloheximide; 80% of this drop was accounted for by an increase in the amount of secreted activity. Cycloheximide added at the beginning of the 37°C incubation blocked accumulation and secretion in X2180 and HMSF 1 (data not shown).

Invertase synthesis and secretion were examined after a change from a repressive (5% glucose) minimal medium to 24°C to a derepressive (2% sucrose plus 0.05% glucose) medium at 37°C (Fig. 2). Secreted invertase activity increased 6.4-fold in X2180 and 1.8-fold in HMSF 1 within 1 hr of the shift, while the intracellular pool remained constant for X2180 but increased 6-fold in HMSF 1. As with acid phosphatase, when HMSF 1 cells were returned to 24°C in the presence of cyclo-

heximide, the intracellular pool of invertase dropped and the secreted activity increased correspondingly.

Cell Division Stops but Protein Synthesis Continues. Cell division and budding in HMSF 1 stopped quickly at 37°C (Fig. 3), with no accumulation at any particular cell cycle position; no increase in cell size was noted. In contrast to cell growth, the rate of protein synthesis in HMSF 1 only slightly decreased in 3 hr at 37°C (Fig. 4), while the rate of protein synthesis in X2180 cells increased. Phospholipid synthesis continued for several hours at 37°C with no significant change in composition (unpublished observations). In the absence of cell growth at 37°C, HMSF 1 cells became dense (16) and phase-refractile, and cell death began after 4 hr (unpublished observations). These properties have been reported for cells undergoing inositol-less death (16). However, in contrast to the sec 1 block, invertase secretion continued during inositol starvation of an auxotrophic strain (unpublished observations).

Thermoreversible Incorporation of a Permease Activity. Incorporation of a sulfate permease activity was used to assess the role of the sec 1 gene product in plasmalemma assembly. Sulfate permease synthesis is derepressed when cells growing in a medium containing 1.5 mM methionine are transferred to a sulfate-free minimal medium (13). Permease activity was first detected in X2180 cells 2 hr after methionine was removed from the medium, and incorporation continued when cells were
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time zero. Aliquote were taken at the times indicated and labeled with
[3H]leucine for 10 min.
small vesicles (0.05-0.07 μm in diameter) in the bud; these
vesicles have been described elsewhere (3, 4). HMSF 1 cells
incubated at 37°C for 1 hr (Fig. 6C) or 3 hr (Fig. 6D and E)
showed a marked accumulation of membrane-bound vesicles,
with no apparent bias toward the bud. The vesicles increased
slightly in size at 37°C, from 0.07-0.09 μm at 1 hr to 0.08-0.10
μm at 3 hr. Cells incubated at 37°C for 1 hr were depleted of
vesicles by growth at 24°C for 3 hr (not shown).
Histochemical staining showed that the accumulated acid
phosphatase was in vesicles (Fig. 7). Strains carrying the sec 1-1
allele and either a constitutive acid phosphatase gene (pho 80,
ACP 1-2; Fig. 7A) or a defective acid phosphatase gene (acp

![FIG. 2. Invertase secretion and accumulation in X2180 (O) and
HMSF 1 (●) cells. Cells were grown in minimal medium with 5% glucose
at 24°C. At time zero, the cells were shifted to a medium with
2% sucrose and 0.05% glucose, and subsequent operations were
performed as indicated. Cycloheximide (100 μg/ml) was added at 1 hr.
Samples were withdrawn, chilled to 0°C, centrifuged, and resus-
pended in cold 10 mM sodium azide. Secreted and intracellular in-
vertase activity was measured. (A) Cells; (B) spheroplast lysate. Three
control experiments were initiated at time zero: in cultures kept at
24°C for 1.5 hr, X2180 had 0.481/0.143 (secreted/intracellular)
units/mg dry weight and HMSF 1 had 0.285/0.162 units/mg; in
cultures shifted from 37° to 24°C with no cycloheximide addition, at 3
hr X2180 had 0.400/0.097 units/mg and HMSF 1 had 0.503/0.114
units/mg; in cultures kept at 37°C with no cycloheximide addition,
at 3 hr X2180 had 0.285/0.089 units/mg and HMSF 1 had 0.155/0.673
units/mg.]

![FIG. 3. Cell division in X2180 (O) and HMSF 1 (●). Cells were
grown in minimal medium at 24°C and shifted to 37°C at time zero,
and aliquots were removed at the times indicated. Parent cells and
buds were counted as separate cells.]

![FIG. 4. Rate of protein synthesis in X2180 (O) and HMSF 1 (●).
Cells were grown in minimal medium at 24°C and shifted to 37°C at
time zero. Aliquote were taken at the times indicated and labeled with
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FIG. 6. Thin-section electron micrographs of X2180 and HMSF 1 cells. Cells were grown in YPD medium and processed for electron microscopy. (A) X2180 cells grown at 37°C; (B) HMSF 1 cells grown at 24°C; (C) HMSF 1 cells warmed to 37°C for 1 hr; (D) HMSF 1 cells incubated at 37°C for 3 hr; (E) higher magnification of D. The horizontal bar is 0.5 µm for A–D and 0.2 µm for E. N, nucleus; Va, vacuole; Ve, vesicle.

1; Fig. 7B) were incubated at 37°C for 2 hr, and the Gamori stain was applied. Although both strains accumulated vesicles, only the phosphatase-containing cells showed staining of the vesicles. Staining of the vacuole in both strains may be due to endogenous phosphate.

DISCUSSION

This report introduces an approach to the study of secretion and surface growth in a eukaryotic cell. Randomly selected temperature-sensitive yeast mutants are screened for the ability to synthesize and secrete two cell-wall enzymes, acid phosphatase...
and invertase. We have assumed that secretion of cell-wall components is necessary for cell viability and that conditional secretory mutants will continue to synthesize protein. Two nonallelic mutants (sec 1-1 and sec 2-1) of the desired phenotype were detected among 87 temperature-sensitive clones. Both mutants accumulate secretory proteins in an intracellular pool at 37°C. Holbein and Kidby (17) succeeded in uncoupling protein synthesis from secretion by proteolytic treatment of yeast spheroplasts, which blocked secretion and allowed a 2-fold increase in the intracellular pool of invertase.

The sec 1-1 mutant influences both secretion and the incorporation of a sulfate permease activity. Sulfate uptake in yeast may require a membrane-bound permease and a periplasmic binding protein, such as has been described for bacterial transport (18). Although transport could be limited by the failure to secrete a binding protein, rather than a defect in permease incorporation, we find that yeast spheroplasts will take up exogenous $^{35}$SO$_4^{2-}$ at 40% of the rate achieved by intact cells (unpublished observation). Thus, if a sulfate-binding protein was required, it would have to adhere to the spheroplast surface. Because of the coordinate accumulation of secretory enzymes and vesicles in HMSF 1 at 37°C and their depletion at 24°C, we propose that the membrane proteins and lipids of the accumulated vesicles are precursors of the plasmalemma and the vesicle soluble contents are precursors of the secreted proteins. This view is supported by the intracellular distribution of a membrane-bound enzyme, chitin synthetase, in HMSF 1. We have previously shown that yeast lysates contain chitin synthetase activity associated with a rapidly sedimenting plasmalemma fraction and a slowly sedimenting microsomal fraction (10). During a 2-hr incubation of HMSF 1 cells at 37°C, the plasmalemma chitin synthetase activity decreased by 50% while the microsomal activity increased 3-fold (unpublished observations).

The function of the sec 1 gene product and the stage in the secretory process that is blocked in the sec 1 mutant are unknown. It is possible that the accumulated vesicles are unable to fuse with the plasmalemma because of defective transport, membrane recognition, or exocytosis. Alternatively, the vesicles may represent an earlier element in the secretory process, such as an intermediate structure between the endoplasmic reticulum and the Golgi apparatus. The behavior of other sec mutants will assist in the genetic, cytological, and biochemical dissection of the secretory pathway in yeast.

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