Proc. Natl. Acad. Sci. USA
Vol. 76, No. 4, pp. 1858–1862, April 1979
Cell Biology

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

(secretory mutants/vesicles/membrane assembly)

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Communicated by Daniel E. Koshland, Jr., January 16, 1979

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 A_{600} 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 A_{600} 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 methanesulfonate, *p*-nitrophenylphosphate, glucose oxidase, *o*-dianisidine, peroxidase, cycloheximide, and homocysteine thiolactone·HCl were from Sigma; $H_2{}^{35}SO_4$, L-[4,5-^{3}H]leucine, L-leucine, and L-methionine were from ICN; mycostatin (nystatin) was from Calbiochem; glusulase was from Endo Laboratories (Garden City, NJ). 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 A_{600} 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 $^{35}SO_4{}^{2-}$ 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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$(NH_4)_2SO_4$ as described by Breton and Surdin-Kerjan (13); units of activity are nmol of SO_4^{2-} uptake per min. Internal acid phosphatase and invertase were assayed in spheroplast lysates. Washed cells (10 A_{600} 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 A_{600} unit of cells and 50 nCi of L-[^{3}H]leucine (0.33 Ci/mol, 1 Ci = 3.70 × 10^{10} 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 A_{600} 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 $CaCl_2$/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 mycostatin 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° 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. 1*B*) 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 in 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 at 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 cycloheximide, the intracellular pool of invertase dropped and the secreted activity increased correspondingly.

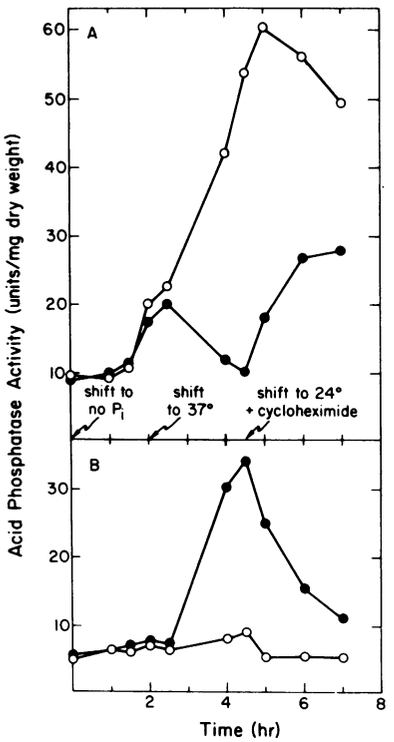

FIG. 1. Acid phosphatase secretion and accumulation in X2180 (○) and HMSF 1 (●) cells. Cells were grown in minimal medium (with 7 mM P_i) at 24°C. At time zero, the cells were transferred to a phosphate-free medium, and subsequent operations were performed as indicated. Cycloheximide (100 μg/ml) was added at 4.5 hr. Samples were withdrawn, chilled to 0°C, centrifuged, and resuspended in cold 10 mM sodium azide. Secreted and intracellular acid phosphatase activity was measured. (*A*) Cells; (*B*) spheroplast lysate. Three control experiments were initiated at time zero: in cultures kept at 24°C for 4.5 hr, X2180 had 124/13.6 (secreted/intracellular) units/mg dry weight and HMSF 1 had 151/15.1 units/mg; in cultures shifted from 37° to 24°C with no cycloheximide addition, at 7 hr X2180 had 118/9.4 units/mg and HMSF 1 had 53.1/7.1 units/mg; in cultures kept at 37°C with no cycloheximide addition, at 7 hr X2180 had 22.7/6.1 units/mg and HMSF 1 had 7.1/19.9 units/mg.

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

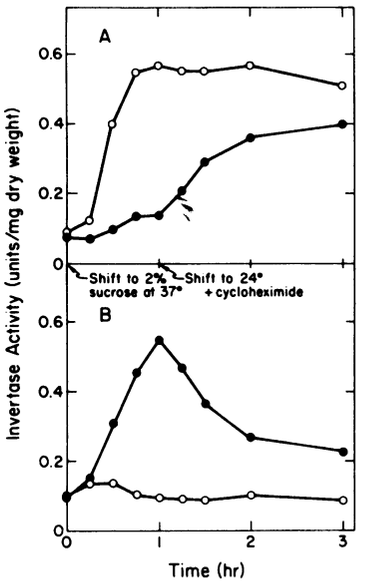

FIG. 2. Invertase secretion and accumulation in X2180 (○) and HMSF 1 (●) cells. Cells were grown in minimal medium with 5% glucose at 24°C. At time zero, the cells were shifted to a medium with 2% sucrose and 0.05% glucose, and subsequent operations were performed as indicated. Cycloheximide (100 μg/ml) was added at 1 hr. Samples were withdrawn, chilled to 0°C, centrifuged, and resuspended in cold 10 mM sodium azide. Secreted and intracellular invertase 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 0.295/0.162 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.

warmed to 37°C (Fig. 5*A*). The permease activity did not appear in HMSF 1 at 37°C, but did appear when cells were returned to 24°C. In a separate experiment, this return of permease activity at 24°C occurred in the presence of cycloheximide (data not shown). The defect was not due to a direct temperature effect on the active permease, because derepressed X2180 and HMSF 1 cells both lost 60% of the permease activity within 0.5 hr of a shift from 24° to 37°C (Fig. 5*B*).

Vesicles Accumulate in Mutant. The intracellular enrichment of secretory enzymes and the reversible block in permease incorporation suggested that HMSF 1 might accumulate an organelle of the secretory apparatus. This suggestion was confirmed when thin sections were examined by electron microscopy. X2180 cells grown at 37°C (Fig. 6*A*) or HMSF 1 cells grown at 24°C (Fig. 6*B*) occasionally showed enrichment of

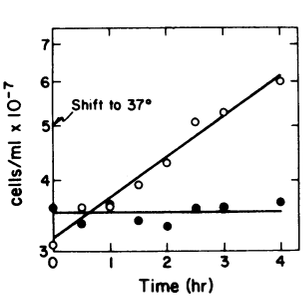

FIG. 3. Cell division in X2180 (○) and HMSF 1 (●). Cells were grown in minimal medium at 24°C and shifted to 37°C at time zero, and aliquots were removed at the times indicated. Parent cells and buds were counted as separate cells.

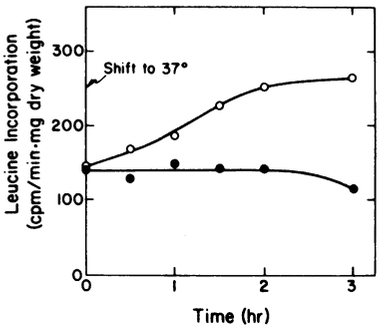

FIG. 4. Rate of protein synthesis in X2180 (○) and HMSF 1 (●). Cells were grown in minimal medium at 24°C and shifted to 37°C at time zero. Aliquots were taken at the times indicated and labeled with [^{3}H]leucine for 10 min.

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. 6*C*) or 3 hr (Fig. 6 *D* 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. 7*A*) or a defective acid phosphatase gene (*acp*

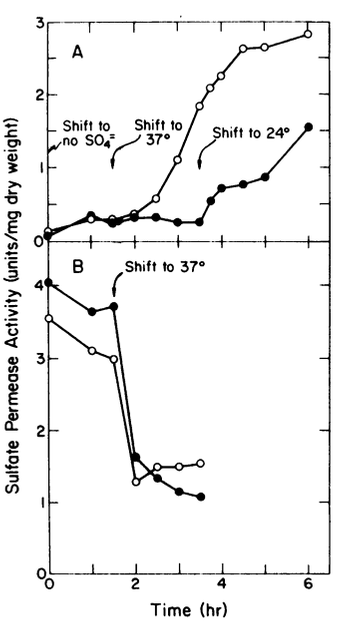

FIG. 5. Incorporation of sulfate permease activity in X2180 (○) and HMSF 1 (●). (*A*) Cells were grown at 24°C in minimal medium supplemented with 1.5 mM methionine and 0.1 mM $(NH_4)_2SO_4$. At time zero, the cells were transferred to a sulfate-free medium, and incubation was continued as indicated in the figure. Samples were withdrawn, chilled to 0°C, centrifuged, and resuspended in cold water. Activity was then measured. Two control experiments were initiated at time zero: in cultures kept at 24°C for 3.5 hr, X2180 had 1.43 units/mg dry weight and HMSF 1 had 1.72 units/mg; in cultures kept at 37°C, at 6 hr X2180 had 1.40 units/mg and HMSF 1 had 0.11 units/mg. (*B*) Cells were grown at 24°C in minimal medium supplemented with 0.25 mM homocysteine thiolactone (derepressing conditions, ref. 13), and the permease activity was monitored in fully derepressed cells for 1.5 hr. The cells were then warmed to 37°C and incubation was continued.

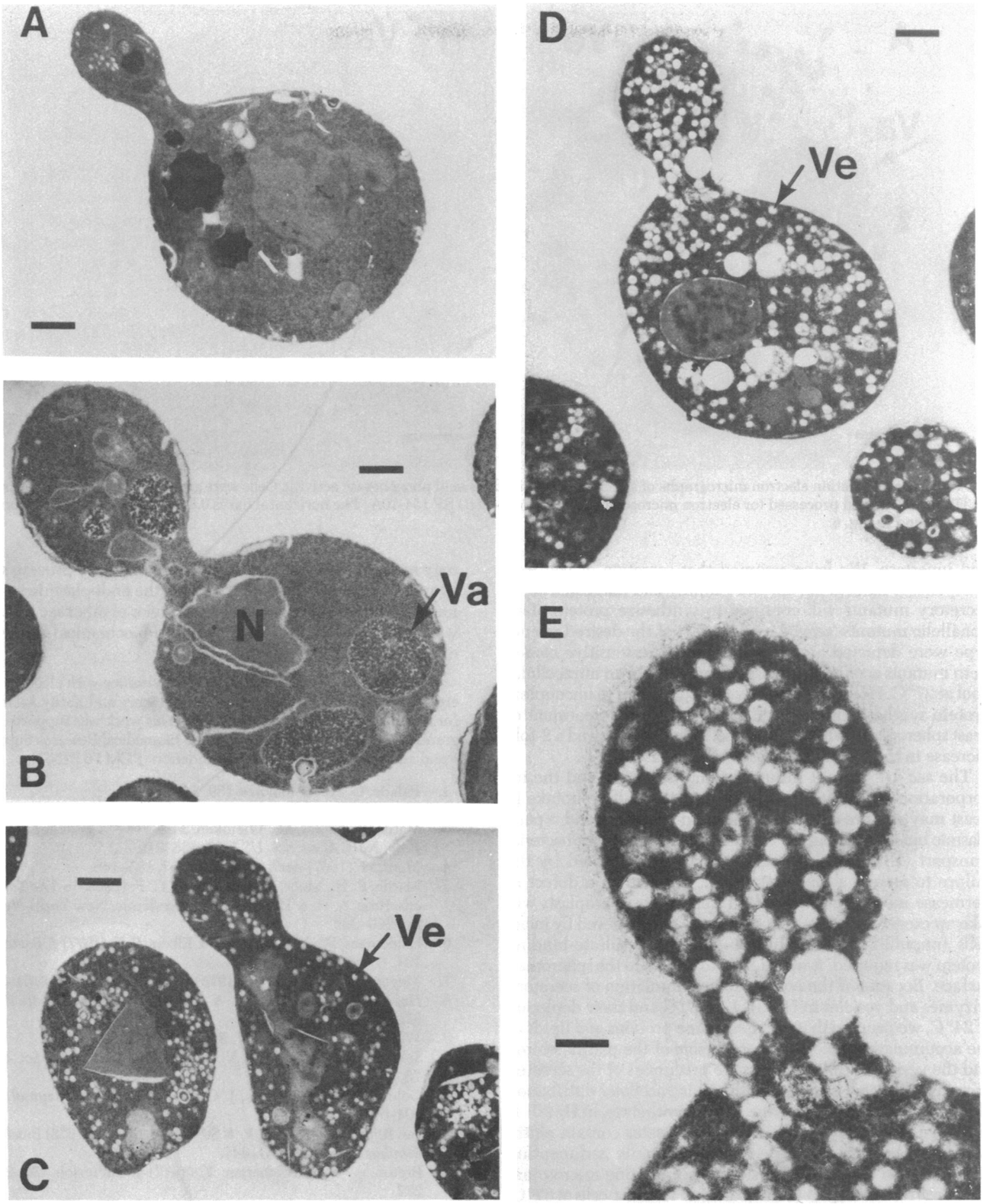

FIG. 6. Thin-section electron micrographs of X2180 and HMSF 1 cells. Cells were grown in YPD medium and processed for electron microscopy. (*A*) X2180 cells grown at 37°C; (*B*) HMSF 1 cells grown at 24°C; (*C*) HMSF 1 cells warmed to 37°C for 1 hr; (*D*) HMSF 1 cells incubated at 37°C for 3 hr; (*E*) 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. 7*B*) 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

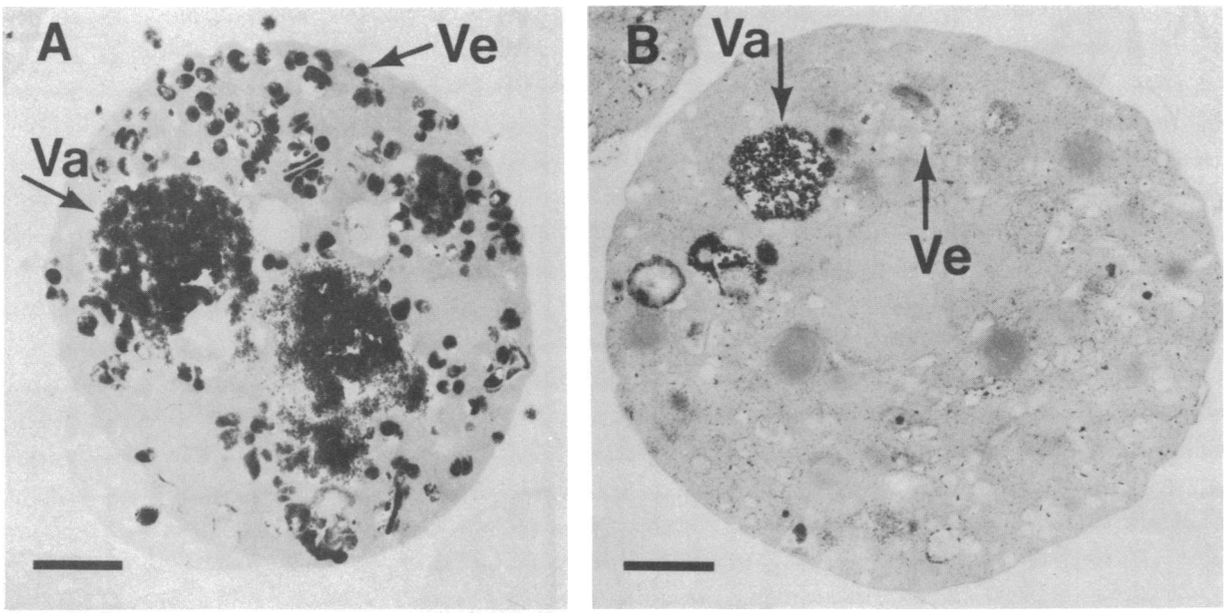

FIG. 7. Thin-section electron micrographs of spheroplasts stained for acid phosphatase activity. Cells were grown in YPD medium, warmed to 37°C for 2 hr, and processed for electron microscopy. (*A*) SF 150-5c; (*B*) SF 154-10A. The horizontal bar is 0.5 μm. Symbols are as described in the legend to Fig. 6.

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, cytologic, and biochemical dissection of the secretory pathway in yeast.

We thank Alice Taylor for her expert assistance with electron microscope techniques. We also thank Susan Henry and Kathy Atkinson for useful discussions during this work. This work was supported by grants from the University of California Biomedical Research Support Fund and the National Science Foundation (PCM 76 21557).

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