

Endocytosis Is Required for the Growth of Vacuolar H⁺-ATPase-defective Yeast: Identification of Six New *END* Genes

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Abstract. Yeast mutants that are defective in acidification of the lysosome-like vacuole are able to grow at pH 5.5, but not at pH 7. Here, we present evidence that endocytosis is required for this low pH-dependent growth and use this observation to develop a screen for mutants defective in endocytosis. By isolating mutants that cannot grow when they lack the 60-kD vacuolar ATPase subunit (encoded by the *VAT2* gene), we isolated a number of *vat2*-synthetic lethal (*Vsl*⁻) mutant strains. Seven of the *Vsl*⁻ mutants are defective in endocytosis. Four of these mutant strains (*end8-1*, *end9-1*, *end10-1*, and *end11-1*) show altered up-

take of the endocytosed ligand, α -factor, and three (*end12-1*, *end12-2*, and *end13-1*) are probably defective in transfer of internalized material to the vacuole. Most of the mutations also confer a strong Ts⁻ growth defect. The mutants defective in uptake of α -factor sort newly synthesized vacuolar proteins correctly, while those which may be defective in subsequent transport steps secrete at least a fraction of the newly synthesized soluble vacuolar proteins. The mutations that result in a defect in α -factor uptake are not allelic to any of the genes previously shown to encode endocytic functions.

ENDOCYTOSIS is a general mechanism that allows eukaryotic cells to internalize both plasma membrane and extracellular material. Experiments with cultured mammalian cells (where endocytosis has been well-characterized at a morphological level) have demonstrated that fluid-phase markers such as horseradish peroxidase or colloidal gold particles are internalized and can be visualized in cell sections by electron microscopy (Hopkins and Trowbridge, 1983; Steinman et al., 1983). After short periods of internalization, the markers are seen in invaginations of the plasma membrane known as coated pits (Anderson et al., 1977; Hopkins and Trowbridge, 1983). These pits bear a coat composed of polymerized clathrin on their cytoplasmic face which has been implicated in the rapid internalization of most, but not all, endocytic markers (Pearse and Robinson, 1990; Hansen et al., 1993). After longer incubation times, the markers label peripheral early endosomes which have a tubuloreticular profile in cell sections and subsequently perinuclear, late endosomes which have a multivesicular structure and which contain mannose 6-phosphate receptors (Helenius et al., 1983; Hopkins and Trowbridge, 1983; Griffiths et al., 1988).

The mechanism of transport of markers from early to late endosomes is unclear. Transport may occur via budding and fusion of transport vesicles or by a maturation process (Griffiths and Gruenberg, 1991; Murphy, 1991). In any event, after prolonged internalization, the bulk of the non-specific markers are localized in lysosomes, which are the major degradative compartments in the cell and are the site where all fluid-phase markers eventually accumulate (Kornfeld and Mellman, 1989). The rate of endocytosis of fluid as estimated using lucifer yellow-carbohydrazide (LY)¹ may vary between cell types, but in murine peritoneal macrophages an estimate is 250 nl/mg of cellular protein/hour (Swanson et al., 1985).

Endocytosis of extracellular fluid by *S. cerevisiae* has been demonstrated using LY. Uptake of LY leads to its accumulation in the vacuole (the yeast equivalent of the mammalian lysosome) and is time-, energy-, and temperature-dependent, consistent with an endocytic mechanism (Riezman, 1985). The rate of endocytosis has been estimated as 27 nl/mg of cellular protein/hour at 30°C (Riezman, 1985). The mating pheromone α -factor is a marker for receptor-mediated endocytosis in yeast and is internalized while bound to

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1. *Abbreviations used in this paper:* CPY, carboxypeptidase Y; 5FOA, 5 fluoroorotic acid; LY, lucifer yellow-carbohydrazide; PBSTM, PBS containing 0.1% Tween 20 and 2% milk powder; PI3-kinase, phosphatidylinositol 3-kinase; Ts, temperature-sensitive; *Vsl*, *vat2*-synthetic lethal.

a specific cell-surface receptor, the *STE2* gene product (Chvatchko et al., 1986; Jenness and Spatrick, 1986; Dulić et al., 1991). Internalized α -factor is transported through two intermediate compartments en route to the vacuole where it is degraded by resident vacuolar proteases (Singer and Riezman, 1990; Singer-Krüger et al., 1993).

Recently, two mutants, *end3-1* and *end4-1*, that are defective for both fluid-phase and receptor-mediated endocytosis, but have no defect in vacuolar protein biogenesis have been isolated and characterized (Raths et al., 1993). Mutations in *END3* or *END4* lead to defects in the internalization step of endocytosis. This step also requires actin and the actin filament-bundling protein, fimbrin (*ACT1* and *SAC6* gene products) (Kübler and Riezman, 1993). Tan et al. (1993) have shown that clathrin heavy chain (*CHC1* gene product) also plays an important role in the internalization step of endocytosis in yeast.

Fewer mutants exist that specifically affect later stages of endocytosis in yeast. Mutations in the *YPT51/VPS21* (Singer-Krüger et al., 1994; Horazdovsky et al., 1994) and *YPT7* (Wichmann et al., 1992; Schimmöller and Riezman, 1993) genes, which encode putative small GTPases of the rab family, specifically affect delivery of internalized material to the vacuole, but also affect vacuole biogenesis. Another mutant, *ren1/vps2*, may also affect a late step in the endocytic pathway (Davis et al., 1993).

The vacuolar system of the yeast cell, and possibly other organelles, are acidified by the action of a membrane-associated H^+ -ATPase which in yeast comprises at least 10

subunits of sizes 102, 95, 69, 60, 54, 42, 36, 32, 27, and 17 kD (Ho et al., 1993; Manolson et al., 1994). The 60-kD subunit is encoded by the *VAT2* gene (Nelson et al., 1989; Yamashiro et al., 1990). In strains bearing a disruption mutation of *VAT2* (*vat2Δ*), the ATPase does not assemble on the vacuolar membrane and consequently the lumen of the vacuole fails to become acidified (Yamashiro et al., 1990). *vat2Δ* strains can grow if the external medium is between pH 5 and pH 6, but are unable to grow if the medium is buffered at pH 7 or above (Nelson and Nelson, 1990; Yamashiro et al., 1990). Nelson and Nelson (1990) have proposed that in the absence of vacuolar ATPase activity, fluid-phase endocytosis may be sufficient to acidify endocytic compartments when the external medium is acidic. This is illustrated in Fig. 1 (see Results). When the external medium is of low pH, the vacuolar system may be acidified either by ATPase-driven H^+ pumping or by endocytosis.

Here we provide experimental evidence that endocytosis is indeed required for growth of *vat2Δ* mutant strains on acidic medium and we have exploited this observation to isolate new *end* mutants that are defective in specific steps of transport from the external environment to the vacuole.

Materials and Methods

Media, Yeast Strains, and Reagents

All the yeast strains described in this report are listed in Table I with their respective genotypes. YPUAD was as described previously (Dulić et al.,

Table I. Yeast Strains Used in This Study

| Strain | Genotype | Source |
|----------|--|-------------|
| RH144-3A | <i>MATα his4 leu2 ura3 bar1</i> | Lab. strain |
| RH144-3D | <i>MATα his4 leu2 ura3 bar1</i> | " |
| RH449 | <i>MATα his4 leu2 ura3 lys2 bar1</i> | " |
| RH977 | <i>MATα his4 leu2 ura3 trp1::URA3 bar1</i> | " |
| RH978 | <i>MATα his4 leu2 ura3 trp1::URA3 bar1</i> | " |
| RH1606 | <i>MATα vat2Δ::LEU2 leu2 ura3 ade6 his4 bar1</i> | " |
| RH1931 | RH1994 × RH1606 diploid | This study |
| RH1937 | <i>MATα vat2Δ::LEU2 leu2 ura3 ade6 his4 bar1/pCY36</i> | " |
| RH1966 | <i>MATα his4 leu2 ura3 lys2 bar1 end4Δ::LEU 2</i> | " |
| RH1972 | <i>MATα vat2Δ::LEU2 leu2 ura3 ade6 his4 end13-1 bar1/pCY36</i> | This study |
| RH1973 | <i>MATα vat2Δ::LEU2 leu2 ura3 ade6 his4 end8-1 bar1/pCY36</i> | " |
| RH1974 | <i>MATα vat2Δ::LEU2 leu2 ura3 ade6 his4 end12-1 bar1/pCY36</i> | " |
| RH1975 | <i>MATα vat2Δ::LEU2 leu2 ura3 ade6 his4 end12-2 bar1/pCY36</i> | " |
| RH1976 | <i>MATα vat2Δ::LEU2 leu2 ura3 ade6 his4 end9-1 bar1/pCY36</i> | " |
| RH1977 | <i>MATα vat2Δ::LEU2 leu2 ura3 ade6 his4 end10-1 bar1/pCY36</i> | " |
| RH1978 | <i>MATα vat2Δ::LEU2 leu2 ura3 ade6 his4 end11-1 bar1/pCY36</i> | " |
| RH1994 | <i>MATα his4 leu2 ura3 bar1 end3Δ::URA3</i> | Lab. strain |
| RH2464 | RH1966 × RH1606 diploid | This study |
| RH2604 | <i>MATα end13-1 leu2 ura3 his4 bar1</i> | " |
| RH2605 | <i>MATα end13-1 leu2 ura3 his4 bar1</i> | " |
| RH2607 | <i>MATα end8-1 leu2 ura3 his4 bar1</i> | " |
| RH2608 | <i>MATα end8-1 leu2 ura3 his4 bar1</i> | " |
| RH2611 | <i>MATα end12-1 leu2 ura3 his4 bar1</i> | " |
| RH2612 | <i>MATα end12-1 leu2 ura3 his4 bar1</i> | " |
| RH2615 | <i>MATα end9-1 leu2 ura3 his4 bar1</i> | " |
| RH2616 | <i>MATα end9-1 leu2 ura3 his4 bar1</i> | " |
| RH2618 | <i>MATα end10-1 leu2 ura3 his4 bar1</i> | " |
| RH2619 | <i>MATα end10-1 leu2 ura3 his4 bar1</i> | " |
| RH2622 | <i>MATα end11-1 leu2 ura3 his4 bar1</i> | " |
| RH2623 | <i>MATα end11-1 leu2 ura3 his4 bar1</i> | " |

1991) except it was supplemented with 40 $\mu\text{g/ml}$ adenine and uracil. For growth of *vat2* Δ mutant strains the pH of the YPUAD was adjusted with HCl to 5 where specified. Minimal medium for plasmid selection was SD (Dulić et al., 1991). Where specified, additional nutrients were added to the SD medium to give a 40- $\mu\text{g/ml}$ final concentration. SD contained one or several of the following seven nutrient supplements when required: adenine, uracil, tryptophan, histidine, lysine, leucine, and tyrosine. The pH of our SD medium was 5.3–5.5.

SD medium containing 5 fluoroorotic acid (5FOA) (1 mg/ml final concentration) (SD/5FOA) was used to select for cells which had been cured of *URA3* plasmids (Boeke et al., 1984). 5FOA (PCR Inc., Gainesville, FL) was added to SD medium to 1 mg/ml final concentration and uracil was added to 50 $\mu\text{g/ml}$ final concentration. Additional supplements were added where required for growth and the pH was adjusted to 5.3–5.5 by addition of 10 M NaOH. The pH of SD/5FOA plates was the same as the SD plates. Where growth on SD/5FOA and SD was directly compared, e.g., during the mutant screen, the SD plates contained 50 $\mu\text{g/ml}$ uracil in addition to the nutrients needed for growth. All solid media contained 2% Bactoagar (Difco, Detroit, MI). SD (low sulfate) and SD (no sulfate) have been described (Raths et al., 1993).

α -Factor internalizations were performed using metabolically labeled [^{35}S] α -factor purified essentially as described by Dulić et al., 1991. The procedure for biosynthetic labeling of α -factor was modified slightly; a 30-min preincubation in SD (no sulfate) was included before the addition of the $\text{Na}_2^{35}\text{SO}_4$ label and labeling was for only 8 h, since this increased the yield of radiolabeled α -factor considerably. The $\text{Na}_2^{35}\text{SO}_4$ (40 mCi/ml) used for metabolic labeling was obtained from Amersham PLC (Amersham, UK).

LY accumulation was assayed using LY obtained from Fluka (Buchs, Switzerland). α -Factor degradation was assessed by separating intact from degraded α -factor on preparative silica gel 60 plates (2.2-mm-thick, 20 \times 20 cm) thin layer chromatography plates supplied by Merck ([Switzerland] AG, Dietikon, Switzerland). Polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970) using 7.5% polyacrylamide/SDS gels. Acrylamide was obtained from BDH (Poole, UK) and sodium dodecyl sulfate was obtained from Sigma Chem. Co., St. Louis, MO. For fluorography, the gels were soaked first in water, and then in 1 M sodium salicylate pH 10 for 1 h, and then dried under vacuum. The gels were exposed to preflashed Kodak XAR X-ray film (Kodak, Rochester, NY) at -80°C and developed using a Fuji automatic film developer.

Yeast Genetic Techniques

Mating of yeast cells, sporulation of diploid strains, tetrad dissection, and scoring of genetic markers was performed as described by Sherman et al. (1974). Transformation of yeast cells was accomplished by the lithium acetate method of Ito et al. (1983). Testing for temperature-sensitive growth was done by streaking yeast cells for single colonies on two YPUAD plates and incubating one plate for 3 d at 24°C and one plate for 3 d at 37°C .

Mutagenesis and Colethality Screen

The strain RH1937, which has the chromosomal copy of the *VAT2* gene disrupted with *LEU2* and carries a YCp50-based plasmid (pCY36; Yamashiro et al., 1990) containing both the *URA3* and the *VAT2* genes, was used for isolation of *vat2*-synthetic lethal (Vsl $^-$) mutants (Table I). The rationale for the screen is shown in Fig. 3. RH1937 cells were mutagenized and mutants that were unable to lose the pCY36 plasmid (thereby uncovering the *vat2* chromosomal mutation) were identified by their inability to grow on SD/5FOA. SD/5FOA selects for cells which do not have a wild-type *URA3* gene; in this case cells that have lost the pCY36 plasmid.

The method used for mutagenizing yeast cells with ethyl methanesulfonic acid (EMS) (Sigma Chem. Co.) was described in Munn et al. (1991). A treatment with EMS which resulted in 30% cell viability was used for the mutant screen. Approximately 7,000 colonies derived from RH1937 cells mutagenized in this way and selected for plasmid retention on SD plates lacking uracil (\sim 200 colonies per plate) were subjected to screening by replica plating onto SD and SD/5FOA selection plates containing uracil. Colonies which grew on SD, but not on SD/5FOA after replica plating were restreaked on both media and incubated at 24°C and 30°C . Those isolates which grew on SD, but gave few or no colonies on SD/5FOA at both temperatures were retained. In this way we isolated 18 mutants.

LY Accumulation Assays

These were performed as described by Dulić et al. (1991). All incubations

were at 24°C for 1 h and the cells were vigorously aerated before incubation. The cells were washed in 1 ml of ice-cold 50 mM sodium phosphate/10 mM sodium azide/10 mM sodium fluoride pH 7.5 (SPAF) four times after incubation with LY. The cells were then resuspended in 30 μl of SPAF and viewed by fluorescence microscopy as described by Dulić et al. (1991).

α -Factor Uptake Assays

[^{35}S] α -factor uptake assays were performed on mid-log phase cells (10^7 cells/ml) using the continuous presence protocol as described by Dulić et al. (1991). The assays were carried out at 24°C and at 37°C with a 15-min preshift before α -factor addition. Internalization was calculated by dividing the internalized counts (pH 1-resistant) by the total cell-associated counts (pH 6-resistant) for each time point, except in the case of the *end10-1* mutant. For the *end10-1* mutant, internalized α -factor was calculated by dividing pH 1-resistant counts at each time point by the pH 6-resistant counts at 5 min. The cell-associated counts decreased very rapidly in this mutant at 37°C , probably due to dissociation of uninternalized α -factor from the receptor.

α -Factor Degradation Assays

[^{35}S] α -factor degradation assays were performed on mid-log phase cells (10^7 cells/ml) using the pulse-chase ([^{35}S] α -factor prebinding for 50 min on ice) protocol as described by Dulić et al. (1991). All assays were performed at 30°C , which is the optimal temperature for α -factor degradation.

Filter Immunoblot for Detection of Secreted CPY

The secretion of CPY from yeast cells during growth was analyzed using the method described by Roberts et al. (1991). Yeast cells were grown on YPUAD plates in contact with a 0.45- μm nitrocellulose filter (Schleicher and Schuell, Dassel, Germany) (overlaid after inoculation of the plate) at 24°C . After incubation in contact with the filter for \sim 40–48 h, the filter was removed from the plate and the adherent yeast cells were eluted with water. The filter was then blocked with PBS containing 0.1% Tween 20 (Merck) and 2% milk powder (PBSTM) for 2 h at room temperature. The filter was then incubated for 3 h with a polyclonal rabbit antiserum against CPY at 1:1,000 dilution in PBSTM. After washing three times in PBSTM, the filter was probed for 1 h with goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma Immunochemicals) at 1:5,000 dilution in PBSTM. Immunoreactive protein bound to the filter was detected by enhanced chemiluminescence (ECL) using the Amersham system and exposure to Kodak XAR x-ray film.

Metabolic Labeling of CPY

Metabolic labeling of cells with $\text{Na}_2^{35}\text{SO}_4$, preparation of spheroplasts, lysis of the spheroplasts, and immunoprecipitation of the labeled CPY from the lysates were all carried out as previously described (Raths et al., 1993) with two modifications. First, the labelings were performed on cells which had been preshifted to 37°C for 15 min. Second, a protease inhibitor cocktail was added to each fraction before addition of SDS and heating of the samples to 90°C . The final concentrations of protease inhibitors were: 0.5 mM PMSF (Sigma Chem. Co.), 1 $\mu\text{g/ml}$ pepstatin (Boehringer-Mannheim Biochemicals, Mannheim, Germany), 1 $\mu\text{g/ml}$ leupeptin (Boehringer-Mannheim). Immunoprecipitates were electrophoresed on 7.5% polyacrylamide/SDS gels and analyzed by fluorography using Kodak XAR X-ray film. The *end8-1* mutant was labeled with [^{35}S]methionine and cysteine (ICN Biochemicals, Irvine, CA) using the method described by Munn et al. (1991), since it was found to label poorly with $\text{Na}_2^{35}\text{SO}_4$ at 37°C .

Results

Yeast Mutants Defective in Both Vacuolar Acidification and Endocytosis Are Inviably

It has been postulated that (a) yeast mutants which have a defective vacuolar H^+ -ATPase may be able to acidify their vacuoles by fluid phase endocytosis if they are grown in acidified medium and (b) that acidification of the vacuole either by H^+ -pumping or by fluid-phase endocytosis of acidi-

fied medium may be essential for growth (Nelson and Nelson, 1990). The hypothesis that yeast cells can acidify their vacuoles by two independent pathways is illustrated schematically in Fig. 1. To test whether yeast strains that are defective in both vacuolar acidification and fluid-phase endocytosis are inviable, we attempted to construct strains carrying mutations in both *VAT2* and *END3*, or in both *VAT2* and *END4*. Yeast cells that carry a *vat2Δ* mutation are inviable at pH 7, but can grow even at 37°C on rich medium between pH 5 and pH 6 (Nelson and Nelson, 1990; Yamashiro et al., 1990). In contrast, both *end3Δ* and *end4Δ* cells are inviable at 37°C (Bénédicti, H., and L. Hicke, personal communication). Two diploid strains, heterozygous for *vat2Δ* and *end3Δ* and for *vat2Δ* and *end4Δ*, respectively, were sporulated, the resulting tetrads were dissected onto rich medium at pH 5, and the haploid spores were allowed to form colonies at 24°C (see Materials and Methods). The growth of haploid colonies from spores derived from the *end3Δ* × *vat2Δ* diploid is shown in Fig. 2. Similar results were obtained from dissection of tetrads from a *end4Δ* × *vat2Δ* diploid (data not shown). The tetrads derived from both strains were of three classes. Approximately 25% of the tetrads contained four viable spores, 50% of the tetrads contained three viable spores, and 25% of the tetrads contained only two viable spores (Table II, A and B), suggesting that two mutations segregated independently in each cross and that the combination of the two mutations was lethal. The total number of spores of each genotype obtained from the *end3Δ* × *vat2Δ* dissection are shown in Table II C. No viable

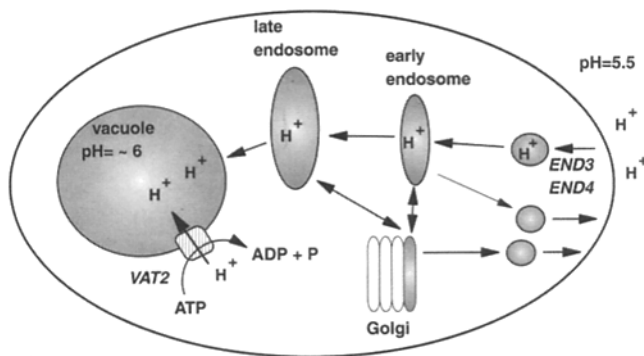


Figure 1. Schematic representation of a yeast cell depicting the endocytic compartments (primary endocytic vesicles, early endosome, late endosome, and vacuole) and the Golgi apparatus. The compartments that may have an acidified lumen are indicated by shading (in some cases the pH is only known for the mammalian counterpart). According to the proposal of Nelson and Nelson (1990) these compartments may be acidified in two ways. (1) Protons may be pumped into the lumen of the acidified organelles from the cytoplasm by the vacuolar H^+ -ATPase (the 60-kD subunit of which is encoded by the *VAT2* gene). (2) Protons may be taken up from the acidified external environment (pH 5.5) by fluid-phase endocytosis, and acidification of the various compartments may occur via transport through the endocytic pathway. In wild-type (*VAT2*) yeast cells the vacuole has been shown to have a pH of 6 (Yamashiro et al., 1990). The site of the endocytic block caused by the *end3Δ* and *end4Δ* mutations is indicated (Raths et al., 1993). A block in endocytosis in conjunction with a defective vacuolar ATPase would be predicted to prevent acidification of the vacuole even when the cells are grown on acidic medium.

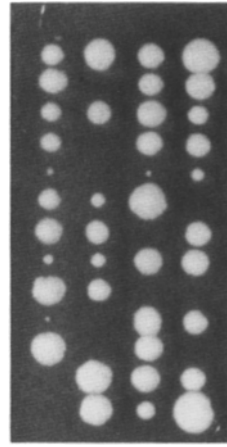


Figure 2. Tetrads derived from a diploid strain heterozygous for both *end3Δ* and *vat2Δ*. The diploid strain (RH1931) was sporulated and tetrads were dissected onto YPUAD medium at pH 5. The spores were allowed to germinate and form colonies at 24°C. Note that many spores did not give rise to colonies. These spores were those that inherited both the *end3Δ* and *vat2Δ* mutations.

vat2Δ endΔ double mutants were recovered. Presumptive *vat2ΔendΔ* spores did germinate in most cases, but gave microcolonies of less than 200 cells in the case of *end3Δ* × *vat2Δ* and less than 20 cells in the case of *end4Δ* × *vat2Δ* (data not shown).

Isolation of Mutants Synthetically Lethal with *vat2Δ*

The observation that both *end3* and *end4* mutations are colethal with a *vat2* mutation at pH 5 indicated that it might be possible to isolate additional *end* mutations by screening for *vat2*-synthetic lethal mutants (Vsl^-), and then testing the Vsl^- mutants for defects in endocytosis. We started with a strain which contains a chromosomal disruption of *VAT2* and a centromere plasmid carrying both *URA3* and the wild-type *VAT2* gene (Table I). This strain is also *MATα* and *bar1* so that it is possible to directly assay the mutants obtained from this strain for defects in α -factor internalization. A screen was conducted (Fig. 3) and retesting of growth on SD and on SD/5FOA at both 24 and 30°C indicated that 18 isolates were most strongly affected for growth on SD/5FOA at both temperatures. Mutants having defects at both 24 and 30°C were thought to be more likely to bear nonconditional mutations and therefore to affect nonessential genes. This rationale was predicted to favor isolation of mutants distinct from previously isolated mutants such as *act1-1*, *end2-1*, and the *sec* mutants which have End^- phenotypes but possess temperature-conditional mutations in essential genes (Novick and Botstein, 1985; Riezman, 1985; Chvatchko et al., 1986). The growth of various Vsl^- mutants on SD and SD/5FOA at 24°C is shown in Fig. 4. Some of the mutants (e.g., 10, 121) gave rise to a few colonies which grew well on SD/5FOA and these presumably arose by mutation at suppressor loci or gene conversion of *vat2Δ* by the plasmid copy of the gene before plasmid loss. The number of these colonies varied from one experiment to another, but most of the cells that lost the plasmid gave microcolonies on SD/5FOA. Cells that are *URA3* (e.g., cells which have not lost the plasmid) do not even form microcolonies on SD/5FOA (data not shown). The inability of the Vsl^- mutants to grow on SD/5FOA was not due to integration of pCY36 into the chromosome or gene conversion of the chromosomal *ura3* mutation, because when mated to the wild-type *ura3 MATα* strain RH449, all the resulting diploids were able to grow on SD/5FOA.

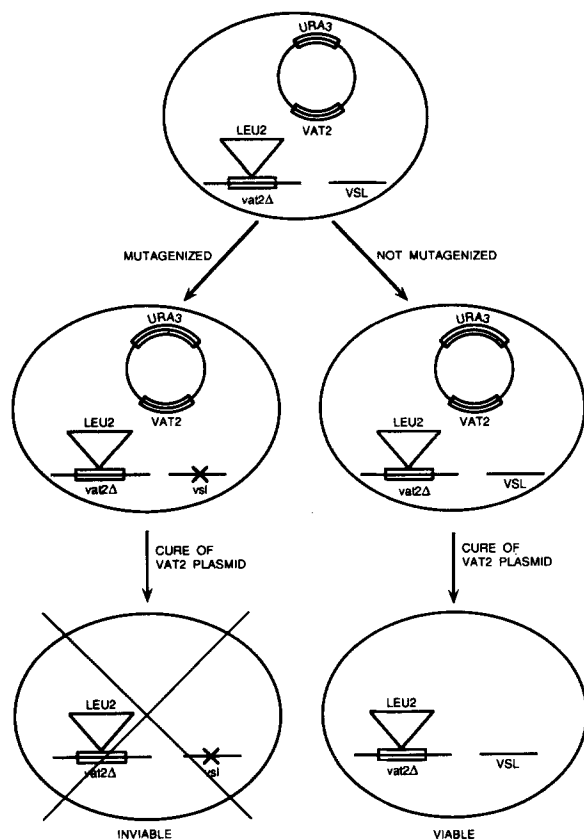


Figure 3. The *vat2*-synthetic lethality (Vsl^-) mutant screen. The starting strain was RH1937, which has a chromosomal deletion of the *VAT2* gene (*vat2Δ*) marked with *LEU2* and a centromere plasmid (pCY36) carrying the wild-type *VAT2* and *URA3* genes. After mutagenesis, some of the cells will gain a new mutation which prevents growth without a wild-type copy of *VAT2* (Vsl^-). SD/5FOA is used to select for those cells in each colony of mutagenized RH1937 which have lost the pCY36 plasmid during growth and become Ura^- . None of the cells in the Vsl^- mutant colonies will be able to grow on SD/5FOA, since they cannot grow without the *VAT2* gene. On the other hand, cells in the Vsl^+ colonies can lose the pCY36 plasmid. Those Vsl^+ cells which do become Ura^- will grow on SD/5FOA.

The results of complementation analysis performed after the first backcross suggested that mutants 38 and 43 are in one complementation group and the other five $End^- Vsl^-$ mutants are in distinct complementation groups. The definition of six complementation groups was confirmed in the complementation analysis based on temperature-sensitivity described later.

Many of the Vsl^- Mutants Are Defective for Lucifer Yellow Accumulation in the Vacuole

We examined the ability of the Vsl^- mutants to carry out fluid-phase endocytosis by assaying the accumulation of the fluid-phase marker LY in the vacuole (Materials and Methods). One of the Vsl^- mutants (113) had no visible vacuole when viewed by Nomarski optics and therefore could not be tested for LY accumulation (data not shown). Each of the other mutants had either a few small vacuoles or one large vacuole (Fig. 5, B-G) similar to the wild-type strain (Fig.

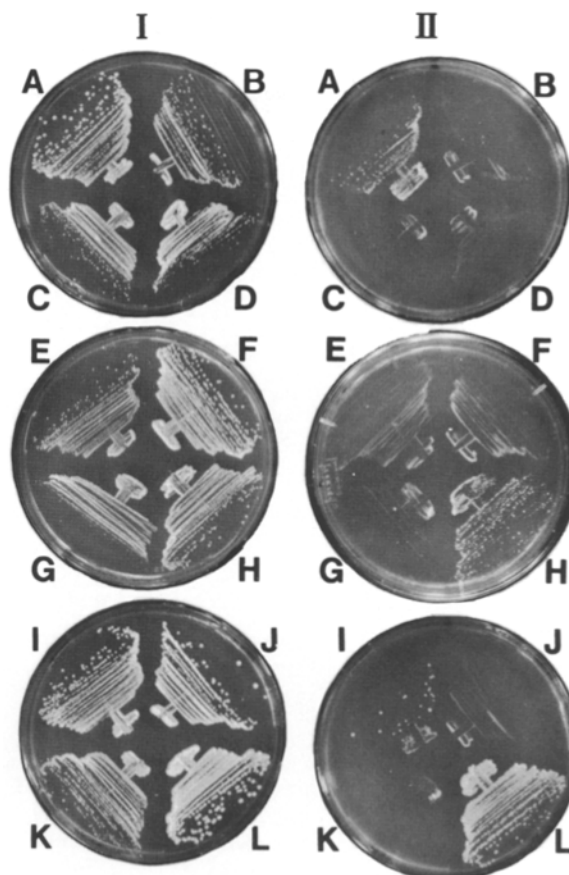


Figure 4. Growth of Vsl^- mutant strains on SD (I), but not on SD/5FOA (II). The mutant strains 10 (RH1972) (A), 19 (RH1973) (B), 38 (RH1974) (C), 43 (RH1975) (D), 72 (E), 95 (F), 113 (G), 121 (H), 62 (RH1976) (I), 73 (RH1977) (J), 82 (RH1978) (K), and the wild-type strain RH1937 (L) were streaked out on SD (pH 5.5) and on SD/5FOA (pH 5.5) plates and allowed to form colonies at 24°C. Only the wild-type parent strain RH1937 (L) is able to form a large number of fast-growing colonies on SD/5FOA plates. Some strains, e.g., RH1972 (A) and 121 (H), occasionally give slow-growing colonies on SD/5FOA plates, but the majority of cells do not grow or form only microcolonies on this medium. Ura^+ cells do not even form microcolonies on SD/5FOA plates. Mutants 10, 19, 38, 43, 62, 73, and 82 are End^- (*end13-1*, *end8-1*, *end12-1*, *end12-2*, *end9-1*, *end10-1*, and *end11-1*, respectively, see Table I), while 72, 95, 113, and 121 are not.

5 A). Mutants 10, 19, 38, 43, 62, 73, and 82 (7 of 18 total) were defective for accumulation of LY in the vacuole at 24°C (Fig. 5, B'-G') compared to the wild-type strain (Fig. 5 A), while most of the other mutants showed approximately wild-type levels of LY accumulation in the vacuole at 24°C (data not shown). The mutant 82 showed a unique staining pattern with LY. In certain cells it was possible to see a faint reticular staining surrounding the vacuole, suggesting that perhaps this mutant took up some LY but did not deliver it to the vacuole. This staining was very faint and is not obvious in Fig. 5.

Those Vsl^- mutants which had defects in endocytosis were designated *end* mutants. Thus mutants 10, 19, 38/43, 62, 73, and 82 define *END13*, *END8*, *END12*, *END9*, *END10*, and *END11*, respectively.

Table II A. Synthetic Lethality of *vat2Δ::LEU2* and *end3Δ::URA3* Mutations

| Tetrad class | PD | T | NPD | Total |
|----------------------|--|--|---|-------|
| Phenotypes of spores | 2 Leu ⁺ Ura ⁻ 2 Leu ⁻ Ura ⁺ | 1 Leu ⁺ Ura ⁻ 1 Leu ⁻ Ura ⁺ 1 Leu ⁻ Ura ⁻ 1 Inviabile | 2 Leu ⁻ Ura ⁻ 2 Inviabile | |
| Genotype of spores | 2 <i>vat2Δ END3</i> 2 <i>VAT2 end3Δ</i> | 1 <i>vat2Δ END3</i> 1 <i>VAT2 end3Δ</i> 1 <i>VAT2 END3</i> 1 <i>vat2Δ end3Δ</i> (inviabile) | 2 <i>VAT2 END3</i> 2 <i>vat2Δ end3Δ</i> (inviabile) | |
| Number of tetrads | 9 | 21 | 9 | 39 |

The diploid strain RH1931 (*vat2Δ::LEU2/VAT2 end3Δ::URA3/END3 ura3/ura3 leu2/leu2*) was sporulated, and the tetrads obtained were dissected onto YPUAD (pH 5) medium. Colonies were allowed to form at 24°C. The nutritional requirements of the haploid segregants arising from the spores, and the deduced genotypes of the haploid segregants with respect to *END3* and *VAT2* were determined. Tetrad ratios for RH1931 (*PD*, *T*, *NPD*) are shown. *PD*, parental ditype; *T*, tetratype; *NPD*, nonparental ditype.

Table II B. Synthetic Lethality of *vat2Δ::LEU2* and *end4Δ::LEU2* Mutations

| Tetrad class | PD | T | NPD | Total |
|----------------------|--|---|---|-------|
| Phenotypes of spores | 4 Leu ⁺ | 2 Leu ⁺ 1 Leu ⁻ 1 Inviabile | 2 Leu ⁻ 2 Inviabile | |
| Genotype of spores | 2 <i>vat2Δ END4</i> 2 <i>VAT2 end4Δ</i> | 1 <i>vat2Δ END4</i> 1 <i>VAT2 end4Δ</i> 1 <i>VAT2 END4</i> 1 <i>vat2Δ end4Δ</i> (inviabile) | 2 <i>VAT2 END4</i> 2 <i>vat2Δ end4Δ</i> (inviabile) | |
| Number of tetrads | 5 | 22 | 9 | 38 |

The diploid strain RH2464 (*vat2Δ::LEU2/VAT2 end4Δ::LEU2/END4 leu2/leu2*) was sporulated, and the tetrads obtained were dissected onto YPUAD (pH 5) medium. Colonies were allowed to form at 24°C. The nutritional requirements of the haploid segregants arising from the spores, and the deduced genotypes of the haploid segregants with respect to *END4* and *VAT2* were determined. Tetrad ratios for RH2464 (*PD*, *T*, *NPD*) are shown. Included in the total are two tetrads which did not fall into *PD*, *T*, or *NPD* classes due to insufficient viable spores recovered or to gene conversion events leading to aberrant marker segregation ratios. *PD*, parental ditype; *T*, tetratype; *NPD*, nonparental ditype.

***end8-1*, *end9-1*, *end10-1*, and *end11-1* Mutants Exhibit Defects in Uptake of α -Factor Pheromone**

Using LY as a marker, it is possible to determine if cells have a defect in endocytosis, but is not possible to show whether the defect lies in uptake at the plasma membrane or in subsequent delivery to the vacuole. To determine where the endocytic defect lies, we assayed each of the new *end* mutants for internalization of α -factor at 24 and 37°C (Materials and Methods). Uptake of α -factor exhibited nearly wild-type kinetics at 37°C in the *end12-1*, *end12-2*, and *end13-1* mutants (Fig. 6, *E* and *F*, data for *end12-2* not shown), but was clearly defective in the *end8-1*, *end9-1*, *end10-1*, and *end11-1* mutants (Fig. 6, *A–D*). Interestingly, uptake of α -factor exhibited wild-type kinetics at 24°C in the *end8-1* mutant (Fig. 6 *A*), and was significantly faster at 24°C than at 37°C in the

end9-1 mutant. α -Factor uptake by the *end11-1* mutant (Fig. 6 *D*) was defective at both temperatures, although the initial rate of uptake (first 5 min) was nearly like that in the wild-type strain. After 15 min at 37°C, the *end11-1* mutant stopped accumulating internalized α -factor as there was no further increase in internal counts. At this time point only ~50–60% of the bound pheromone was resistant to an acid wash. The defect in α -factor uptake shown by the *end10-1* mutant (Fig. 6 *C*) was similar to that exhibited by the *end11-1* mutant, except that in the former mutant the noninternalized α -factor dissociated rapidly from the cells.

The *end12-1*, *end12-2*, and *end13-1* Mutants Are Defective in Degradation of Internalized α -Factor

To measure later steps of endocytosis, we tested those *end* mutants which were defective for LY accumulation but were not defective for α -factor uptake, for α -factor degradation at 30°C (Materials and Methods). We chose 30°C because pheromone delivery to the vacuole is optimal at this temperature. The results of these experiments are shown in Fig. 7. The *end13-1* mutant showed a strong delay in degradation of internalized α -factor at 30°C compared to the wild-type strain and the *end12-1* (and *end12-2*) mutants show almost no degradation of the internalized α -factor even at late time points (90'). The *end11-1* mutant also showed very little degradation of the small amount of internalized α -factor (data not shown).

The *end12-1* and *end13-1* Mutants Missort Newly Synthesized Vacuolar Hydrolases

In yeast, soluble vacuolar proteins transit through endosomal compartments on their way to the lysosome or vacuole (Schimmöller and Riezman, 1993; Raymond et al., 1992; Vida et al., 1993; Davis et al., 1993). It is therefore possible that certain mutations that block or delay transport between endosomal compartments or from endosomal compartments to the vacuole might cause a defect in the correct sorting and maturation of soluble vacuolar hydrolases. We therefore screened the new *end* mutants for secretion of carboxypeptidase Y (CPY), a soluble glycoprotein of the yeast vacuole. The *end* mutants were grown on a nitrocellulose filter at

Table II C. Synthetic Lethality of *vat2Δ::LEU2* and *end3Δ::URA3* Mutations

| Phenotype Genotype | Ura ⁻ Leu ⁻ <i>END3 VAT2</i> | Ura ⁻ Leu ⁺ <i>END3 vat2Δ</i> | Ura ⁺ Leu ⁻ <i>end3Δ VAT2</i> | Ura ⁺ Leu ⁺ <i>end3Δvat2Δ</i> | Total |
|-----------------------|---|--|--|--|-------|
| Number observed | 36 | 40 | 41 | 0 | 117 |
| Number Case 1 | 39 | 39 | 39 | 0 | 117 |
| Number Case 2 | 39 | 39 | 39 | 39 | 156 |

The total number of spores of each genotype obtained by dissection of 39 tetrads of RH1931 are shown. The data is from the experiment described in Table II A. The number expected if *end3Δ* and *vat2Δ* are unlinked and show synthetic lethality (Case 1), and if *end3Δ* and *vat2Δ* are unlinked and do not show synthetic lethality (Case 2) are also shown.

24°C and CPY secreted by the cells was detected using a specific polyclonal antiserum (Materials and Methods). The result is shown in Fig. 8. The *end12-1* and *end13-1* mutants were positive for the presence of CPY on the filter and the *end8-1*, *end9-1*, and *end11-1* mutants were negative as was the wild-type strain. The results obtained with the *end10-1* mutant were intermediate and probably reflect some cell lysis (see below).

To further analyze delivery of CPY to the vacuole, we performed a pulse-chase radiolabeling of newly synthesized CPY in each *end* mutant. CPY is synthesized as a precursor (prepro-CPY) with an NH₂-terminal signal sequence and is translocated into the lumen of the ER. In the ER, CPY receives four core oligosaccharides to yield a protein of 67 kD known as p1 CPY. CPY is then transported to the Golgi where it receives outer chain mannose residues and increases in size to 69 kD (p2 form). From the Golgi, the CPY is transported to the vacuole where it is cleaved to a 61-kD form which is the mature, active species. The formation of 61 kD CPY is indicative of correct vacuolar delivery of CPY (for review see Rothman et al., 1989; Klionsky et al., 1990).

The new *end* mutants were pulse-labeled with Na₂³⁵SO₄ or [³⁵S]methionine and cysteine, chased with cold sulfate, methionine, and cysteine, and separated into intracellular and extracellular (including periplasmic) fractions. Labeled newly synthesized CPY was immunoprecipitated at various time points after initiation of the chase from both fractions and displayed on SDS-PAGE gels (Fig. 9). The *end8-1*, *end9-1*, *end10-1*, *end11-1*, and *end13-1* mutants exhibited wild-type kinetics of CPY maturation in the intracellular fractions whereas the *end12-1* mutant was severely defective for maturation of CPY. Although the *end13-1* mutant matured intracellular CPY with wild-type kinetics, a small amount of the newly synthesized CPY was secreted as p2 CPY into the extracellular fraction. Thus, the pulse-chase labeling data agrees with the filter immunoassay data, and shows that only *end12-1* (and *end12-2*) and *end13-1* have defects in delivery of CPY to the vacuole. Since these mutants were also defective in degradation of internalized α -factor, this result is consistent with the idea that delivery of newly synthesized CPY to the vacuole and late steps in endocytosis require common gene products. Further work will be needed to establish that the α -factor degradation phenotypes of these mutants are really due to transport blocks and are not due to a lowered ability of the vacuoles in these mutants to degrade α -factor.

All of the New *end* Mutants Affect Nuclear Genes and Display Defective Growth at Elevated Temperatures

All of the new *end* mutants (with the exception of *end12-2*,

which is allelic to *end12-1*) were outcrossed to the congenic wild-type strains RH449 (once) and RH977 or RH978 (twice). The defect in uptake of LY segregated 2:2 in every tetrad examined, indicating that the End⁻ phenotype is caused by a lesion in a nuclear-encoded gene. In addition, an unselected defect in growth at elevated temperature segregated with each endocytic defect. The *end8-1*, *end9-1*, *end10-1*, and *end12-1* (and *end12-2*) mutants caused a tight block in growth at 37°C, whereas the *end11-1* strain grew poorly at all temperatures tested (24 and 37°C) and the *end13-1* strain was only completely defective for growth at 39°C (the wild-type strain grew slowly at this temperature). The Ts⁻ in each *end* mutant was complemented in heterozygous diploids. Hence the Ts⁻ phenotype which cosegregates with each *end* mutation is recessive.

Other mutants have been isolated in our laboratory and in other laboratories that affect the internalization step of endocytosis. Among these are *chcl-521* (clathrin heavy chain) (Payne et al., 1988; Tan et al., 1993), *clclΔ* (clathrin light chain) (Silveira et al., 1990), *act1-1* (actin) (Novick and Botstein, 1985; Kübler and Riezman, 1993), *sac6Δ* (fimbrin) (Adams et al., 1991; Kübler and Riezman, 1993), *cmd1-1* (calmodulin) (Davis, 1992; Kübler, E., F. Schimmöller, and H. Riezman, manuscript in preparation), *end3-1* (Raths et al., 1993), *end4-1* (Raths et al., 1993), *end5-1*, *end6-1*, and *end7-1* (Munn, A. L., and H. Riezman, unpublished data). All of these mutants, like the new *end* mutants, exhibit temperature-sensitive growth. We tested each of the new *end* mutants for complementation with these mutants. All pairwise combinations exhibited complementation. All the mutations were recessive to the wild-type allele in heterozygous diploids (data not shown). Hence, the new *end* mutants isolated in the Vsl⁻ screen that are defective in the internalization step identify a new set of genes required for fluid-phase and receptor-mediated endocytosis in yeast.

The *end12* and *end13* mutants, which show a vacuolar protein sorting (*vps*) phenotype, were tested by complementation analysis with the *vps* mutant collection (Raymond et al., 1992). *end12* did not complement *vps34*, and *end13* did not complement *vps4*. Therefore, the new screening procedure also identifies a subset of *vps* mutants that affect the endocytic pathway (see Discussion).

Discussion

In previous work, two yeast mutants blocked in the first step of endocytosis were isolated. These mutants carried lesions in distinct genes, *END3* and *END4* (Raths et al., 1993). In this study, we have shown that the combination of *end3Δ* or *end4Δ* mutations with a *vat2Δ* mutation resulted in spores

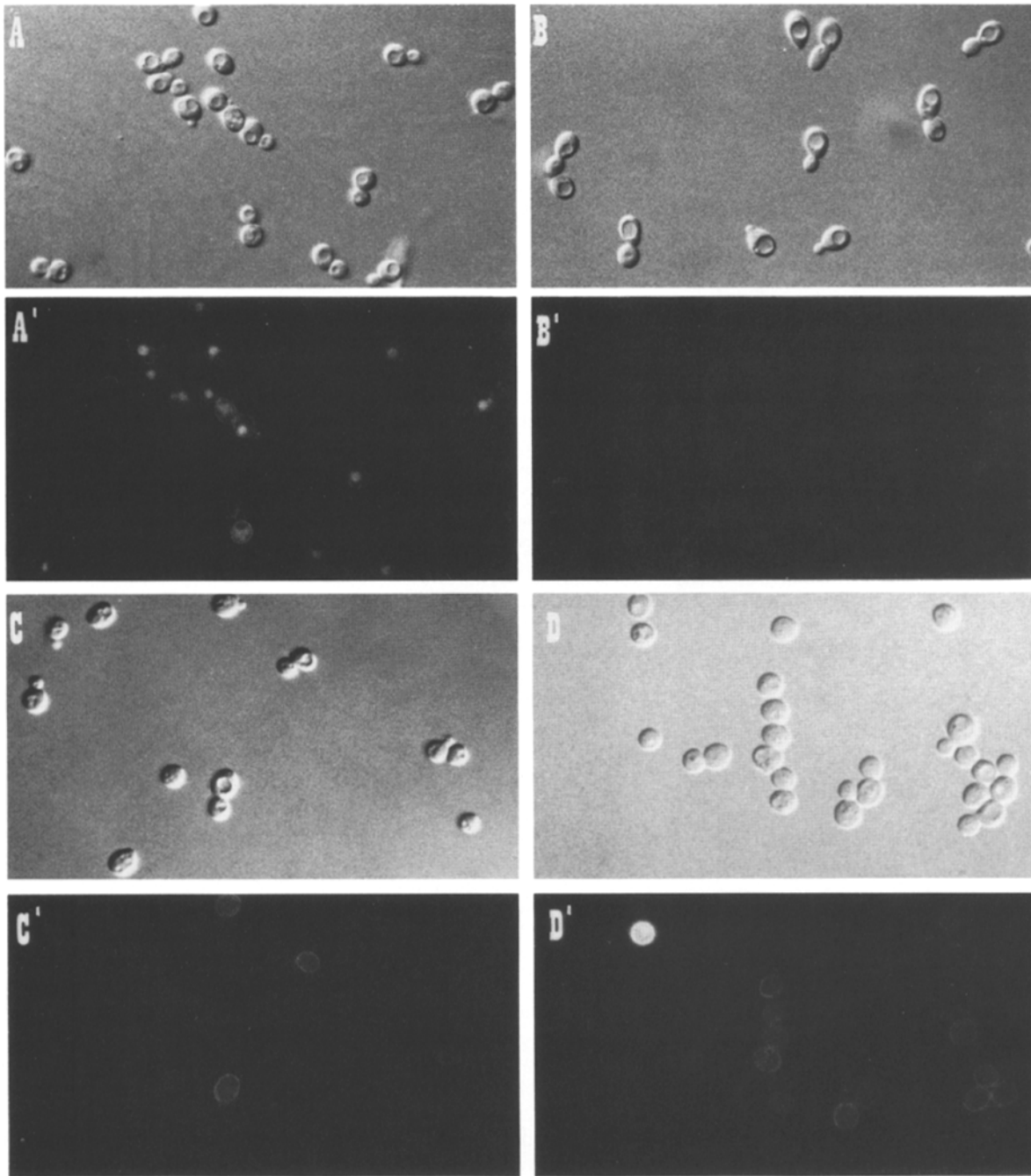


Figure 5. Some *Vsl⁻* mutants are defective for accumulation of LY in the vacuole (End^-). The wild-type strain RH144-3D (*END*) (A) and the three time outcrossed mutants 10 (*end13-1*) (RH2604) (B), 19 (*end8-1*) (RH2607) (C), 38 (*end12-1*) (RH2611) (D), 62 (*end9-1*) (RH2615) (E), 73 (*end10-1*) (RH2618) (F), and 82 (*end11-1*) (RH2622) (G) were assayed for accumulation of LY in the vacuole at 24°C as described in Materials and Methods. The upper panel in each pair (A–G) shows a field of cells viewed with Nomarski optics, and the lower panel in each pair (A'–G') shows the same field of cells viewed with FITC-fluorescence optics. The vacuoles in each cell are visible as indentations when observed with Nomarski filters. If the vacuoles have accumulated LY, then they are also visible with fluorescence optics. All the mutants shown have vacuoles which are clearly visible in the Nomarski photographs, but which are unstained or stained poorly with LY compared to the wild-type strain in the fluorescence photographs. The original mutants RH1972 (*end13-1*), RH1973 (*end8-1*), RH1974 (*end12-1*), RH1975 (*end12-2*), RH1976 (*end9-1*), RH1977 (*end10-1*), and RH1978 (*end11-1*) have similar defects in LY accumulation.

that germinated but did not give rise to colonies. Therefore, it seems that in the absence of the normal mechanism for acidification of the vacuolar system, endocytosis of acidic, external fluid can allow *vat2Δ* mutant cells to grow. When these cells are also unable to endocytose, the vacuolar sys-

tem cannot be acidified and this leads to lack of growth consistent with the hypothesis put forward by Nelson and Nelson (1990). It is quite possible that the rate at which the *vat2Δ* cells acidify their vacuolar system by endocytosis is limiting for growth and is responsible for their slow generation time.

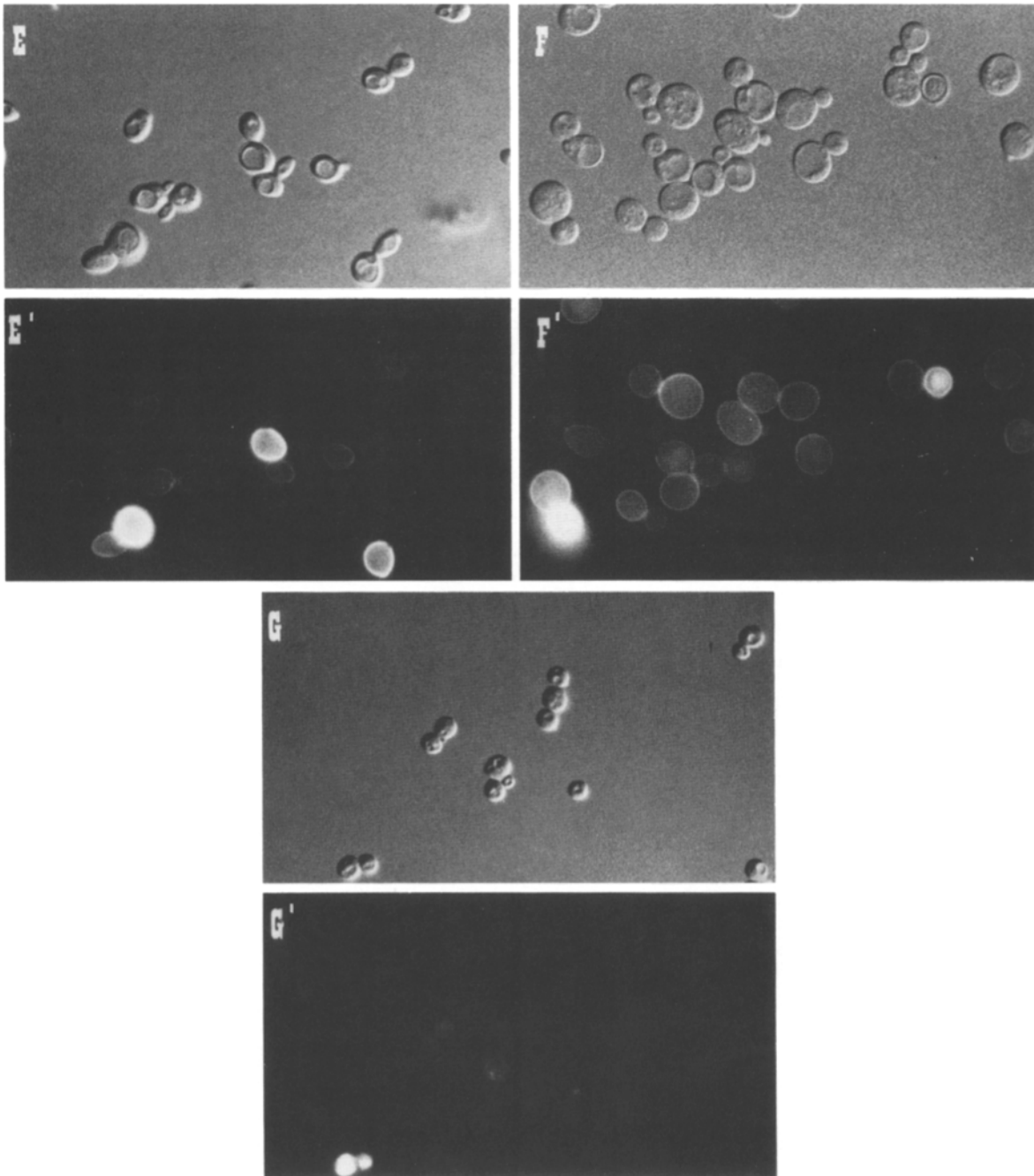
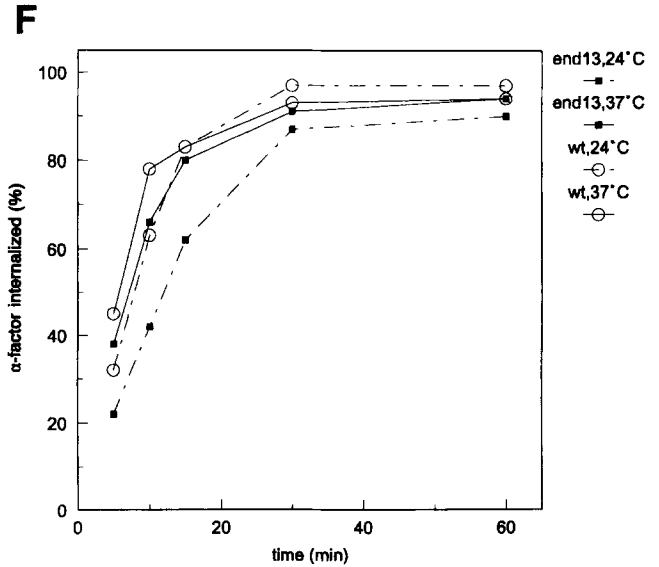
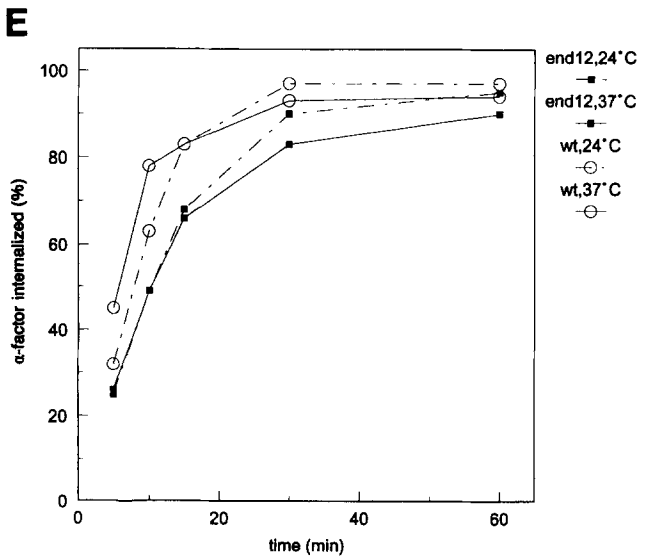
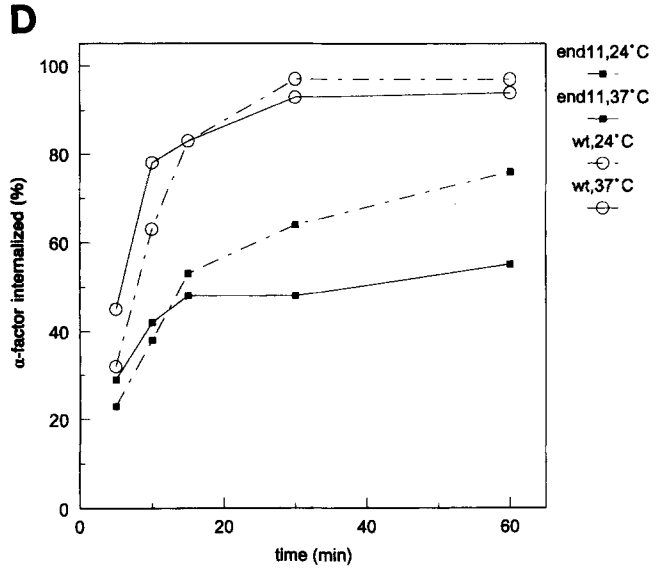
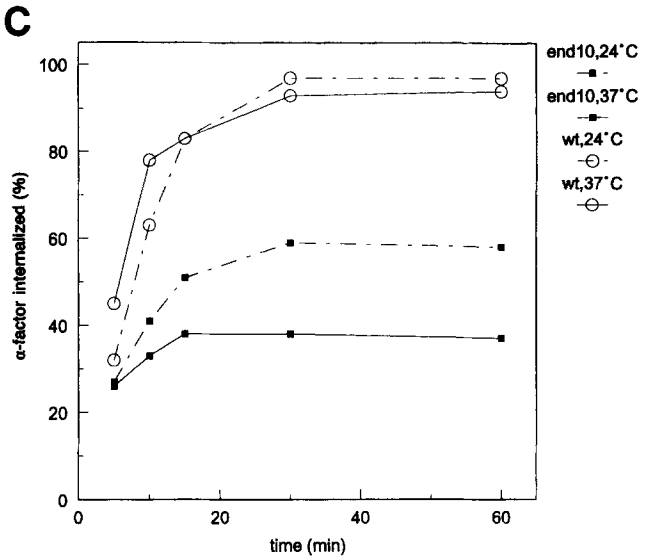
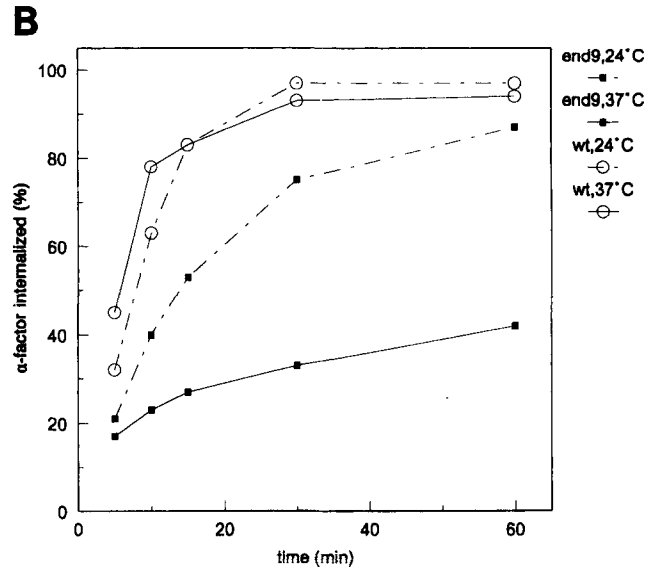
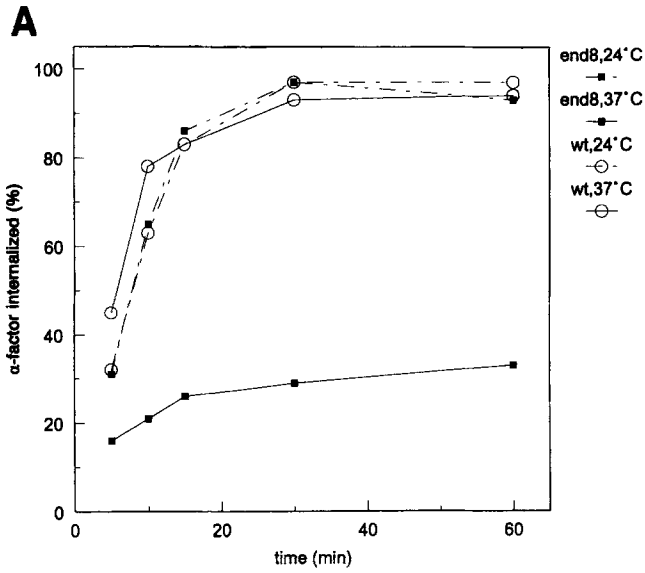


Figure 5.

The experiments described in this report suggest the possibility that an organelle(s) of the vacuolar or secretory system must be acidified to allow growth. Further work will be needed to determine whether or not this is true. If a compartment is being acidified via endocytosis, however, several observations may be useful clues to the identity of this compartment. First, not all mutations that affect accumulation of LY in the vacuole are synthetically lethal with *vat2* mutations. In particular, the *ypt7* mutation, which delays endocytic traffic from late endosomes to vacuoles, is not synthetically lethal with *vat2* (Schimmöller and Riezman, 1993; Schimmöller, F., unpublished observations). On the other hand, some mutants that affect postinternalization stages of en-

docytosis, e.g., the *endl2-1*, *endl2-2*, and *endl3-1* mutants described here, are synthetically lethal with *vat2*. This suggests that if uptake of the acidic medium is important for growth, uptake alone is not sufficient and its delivery to some internal compartment(s) of the endocytic or perhaps secretory pathway is also required. In any event, it is unlikely that the vacuole must be acidified, because it is known that some yeast mutants remain viable without functional vacuoles (Dulic and Riezman, 1990). Further experiments will be required to determine if there is an organelle whose acidification is essential and, if so, the identity of this organelle.

By screening for mutations that are co-lethal with *vat2*Δ



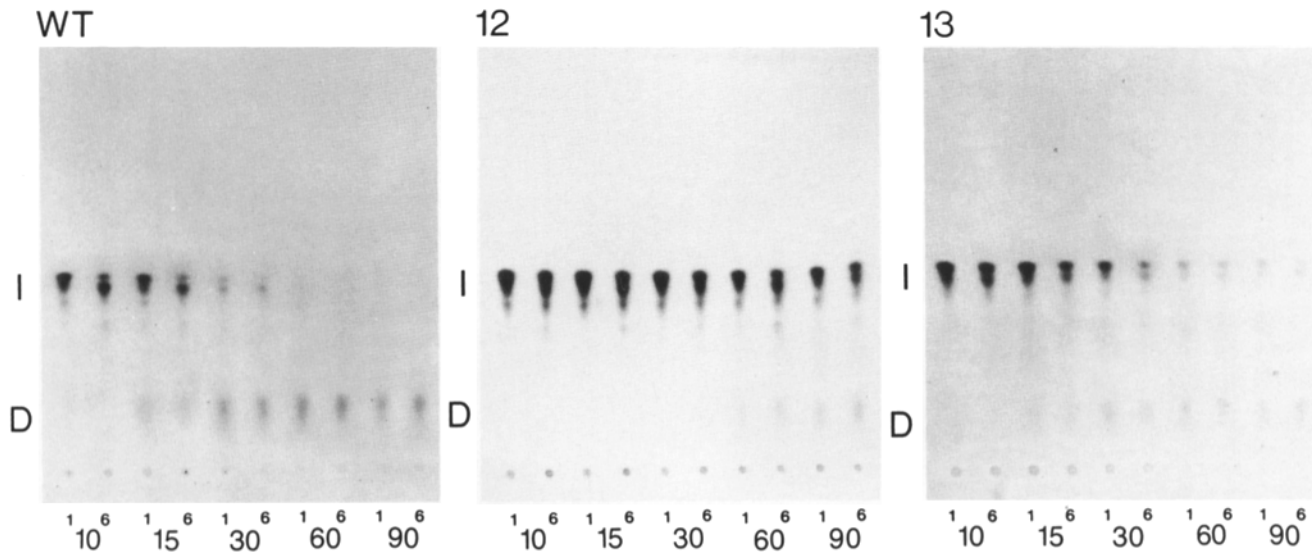


Figure 7. The *End*⁻ mutants which are wild-type for α -factor uptake (10/*endl3-1* and 38/*endl2-1*) are defective for α -factor degradation. The three time outcrossed mutants 10 (*endl3-1*) (RH2604) (13) and 38 (*endl2-1*) (RH2611) (12) and the wild-type strain (RH144-3D) (WT) were assayed for α -factor degradation using a pulse-chase protocol as described in Materials and Methods. Mutant 10 (*endl3-1*) degrades the α -factor with slow kinetics, while mutant 38 (*endl2-1*) is nearly completely blocked in α -factor degradation. Quantification of the intact α -factor remaining at different time points by laser densitometry (using the fluorograms shown) indicates that in RH144-3D only 34% of the internalized α -factor is intact at 30 min, while in RH2604 and RH2611, 70% and 93% of the internalized α -factor is intact at 30 min, respectively. By 90 min, only 7% of the internalized α -factor is intact in RH144-3D, while at the same time 16% and 64% of the internalized α -factor is intact in RH2604 and RH2611, respectively.

(*Vsl*⁻), we isolated seven new mutants that have endocytic defects. These mutants define six complementation groups (*END8-END13*). Therefore, it seems likely that more genes required for endocytosis will be identified by further screens for *Vsl*⁻ mutants. Of the new mutants, four affect uptake of endocytic markers into the cell (*end8-1*, *end9-1*, *endl0-1*, and *endl1-1*) and three may affect subsequent delivery of internalized material to the vacuole (*endl2-1*, *endl2-2*, and *endl3-1*). The uptake-defective mutants are able to deliver and mature the soluble vacuolar protein CPY with wild-type kinetics and do not secrete this protein to the cell surface. In this respect, they resemble *end3* and *end4* mutants. In contrast, the three mutants which may affect subsequent steps in the endocytic pathway (*endl2-1*, *endl2-2*, and *endl3-1*) are defective for delivery of newly synthesized CPY to the vacuole. At least a portion of the newly synthesized CPY in these latter mutants reaches the extracellular medium in the p2 form.

The secretion of vacuolar hydrolases by *endl2* and *endl3* mutant yeast cells makes the interpretation of the α -factor degradation experiments more difficult. Perhaps these mutants are defective in degradation of the internalized α -factor because of a deficiency in vacuolar protease activity and not because of a delay in delivery of α -factor to the vacuole. We

believe that there is a delay in α -factor transport to the vacuole because the accumulation of LY in the vacuole is also blocked in these mutants. Accumulation of LY in the vacuole does not require vacuolar proteases and several *vps* mutants which we have tested, including *vps1* and *vps3* which have strong CPY missorting phenotypes (Raymond et al., 1992), accumulate LY in the vacuole as well as wild-type cells (data not shown). The slow degradation of α -factor in the *endl3-1* mutant may be consistent with a strong delay in transport of α -factor to the vacuole. This slow degradation is reminiscent of that seen in *ypt7* mutant yeast and may reflect degradation in a prevacuolar/endosomal compartment in which newly synthesized vacuolar hydrolases accumulate (Schimmöller and Riezman, 1993). In future experiments we will use cell fractionation techniques to follow the intracellular distribution of α -factor at various times after internalization. These experiments will allow us to determine whether there is a transport block in the endocytic pathway in the *endl2/vps34* and *endl3/vps4* mutants and which steps in the endocytic pathway are affected. If there is a defect in transport of α -factor to the vacuole in these mutants, we should be able to identify the compartment in which the α -factor accumulates.

The isolation of alleles of *vps4* and *vps34* in our mutant

Figure 6. Some of the *End*⁻ mutants are defective in α -factor internalization. Continuous presence α -factor uptake assays were performed at 24°C and at 37°C as described in Materials and Methods on the wild-type strain RH144-3D (*END*) (shown in each graph A-F) and the three time outcrossed mutants 19 (*end8-1*) (RH2607) (A), 62 (*end9-1*) (RH2615) (B), 73 (*endl0-1*) (RH2618) (C), 82 (*endl1-1*) (RH2622) (D), 38 (*endl2-1*) (RH2611) (E), and 10 (*endl3-1*) (RH2604) (F). Mutants 19 (*end8-1*) and 62 (*end9-1*) (A and B) are defective in internalization even at early time points at 37°C, but uptake is less defective (or not defective) at 24°C. Mutants 73 (*endl0-1*) and 82 (*endl1-1*) (C and D) internalize α -factor like wild-type initially, but then uptake slows down when ~40–50% of the α -factor is internal, i.e., after 10–15 min. Mutants 10 (*endl3-1*) and 38 (*endl2-1*) (E and F) internalize α -factor like wild-type at 24°C and 37°C. Data shown is the average of at least four independent assays.

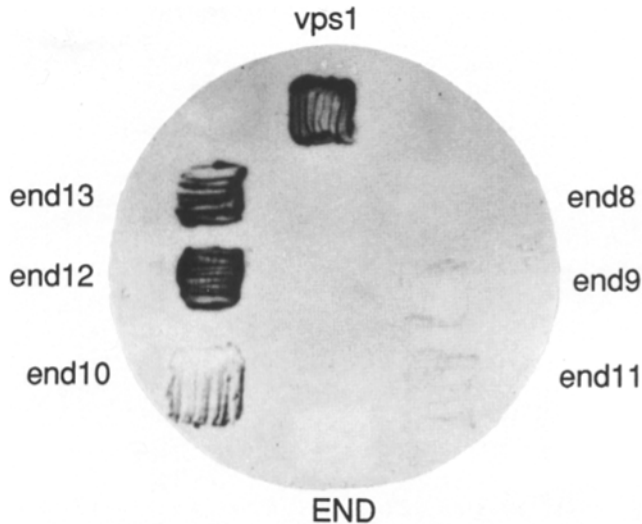


Figure 8. The *End*⁻ mutants which are defective for α -factor degradation secrete soluble vacuolar proteases. Three time outcrossed mutants 10 (*end13-1*) (RH2604), 19 (*end8-1*) (RH2607), 38 (*end12-1*) (RH2611), 62 (*end9-1*) (RH2615), 73 (*end10-1*) (RH2618), and 82 (*end11-1*) (RH2622) as well as the wild-type strain RH144-3D (*END*) and the *vps1* strain as a positive control were grown on a nitrocellulose filter at 24°C. After two days of growth, the cells were washed from the filter and the filter was probed with antiserum raised against carboxypeptidase Y (CPY) to visualize CPY which had been secreted during growth (see Materials and Methods). The *end12-1* and *end13-1* mutants are strongly positive, as is the *vps1* strain. The *end10-1* mutant is faintly positive. The *end8-1*, *end9-1*, and *end11-1* mutants are negative like the wild-type strain RH144-3D.

screen is significant. The *vps4* mutants are of the class E type as described by Raymond et al. (1992). They have vacuoles of wild-type morphology, however soluble vacuolar proteins such as CPY accumulate in a compartment adjacent to, but distinct from, the vacuole. This "class E compartment" is acidified and the vacuolar H⁺-ATPase is associated with it. Raymond et al. (1992) have suggested that it may be an endosomal/prevacuolar intermediate lying between the Golgi complex and the vacuole. It is notable also that the *ren1* mutant isolated by Davis et al. (1993), which exhibits a delay in uptake and/or transport of the α -factor receptor (Ste3p) is allelic to *vps2*, another class E *vps* mutant.

In contrast to *vps4*, *vps34* mutants are of the class D type. Class D *vps* mutants have vacuoles that are often larger than those seen in wild-type cells (Raymond et al., 1992). Nevertheless, class D mutants have stronger defects in growth at elevated temperature, growth in high osmolarity medium, and in vacuolar protein sorting compared to class E *vps* mutants. The vacuole is not strongly acidified in these mutants and the vacuoles are not able to segregate efficiently into daughter cells during mitosis (Banta et al., 1988; Raymond et al., 1992).

A better understanding of the role of the *VPS34* gene product in vacuolar protein sorting has been gained by the discovery that it possesses a phosphatidylinositol 3-kinase (PI3-kinase) activity and has amino acid homology to the catalytic subunit (p110) of mammalian PI3-kinase (Hiles et al., 1992; Herman et al., 1992). In mammalian cells, PI3-kinase is as-

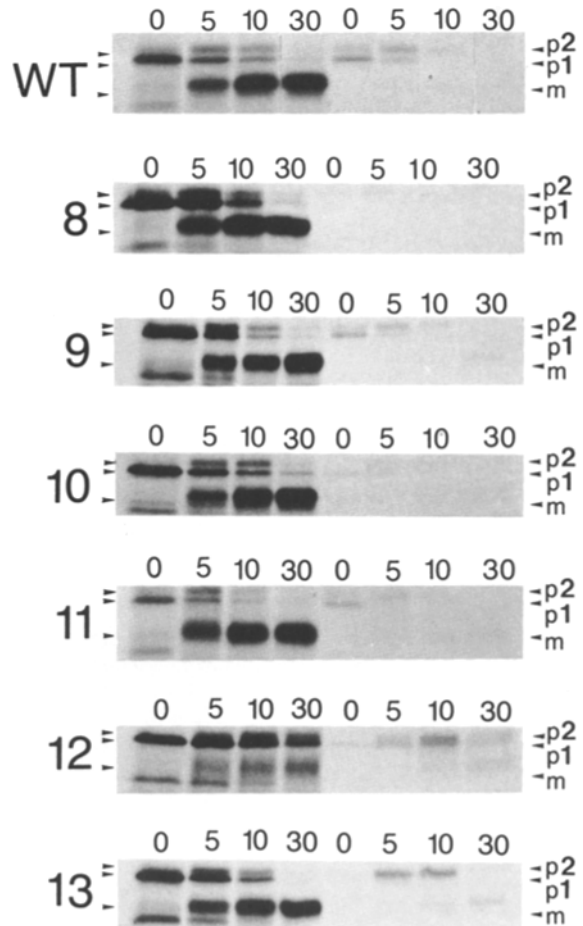


Figure 9. All the new *End*⁻ mutants except 38 (*end12*) process soluble vacuolar protease precursors like the wild-type strain RH144-3A. Three time outcrossed mutants *end8-1* (mutant 19) (RH2608) (8), *end9-1* (mutant 62) (RH2616) (9), *end10-1* (mutant 73) (RH2619) (10), *end11-1* (mutant 82) (RH2623) (11), *end12-1* (mutant 38) (RH2612) (12), *end13-1* (mutant 10) (RH2605) (13) as well as the *END* strain (RH144-3A) (*WT*) were metabolically labeled with Na₂³⁵SO₄ or with [³⁵S]methionine and -cysteine at 37°C, and then chased with cold Na₂³⁵SO₄, methionine, and cysteine at the same temperature. After various times of chase (0, 5, 10, and 30 min), samples of cells were removed and protein transport was terminated with azide and fluoride. The samples were separated into cellular and extracellular fractions and CPY was immunoprecipitated from each fraction. The immunocomplexes were resolved on SDS-PAGE gels and the immunoprecipitated CPY was visualized by fluorography. The cellular fractions from each time point are shown on the left, and the extracellular fractions from each time point are shown on the right. *p1* is the core-glycosylated (endoplasmic reticulum) form of CPY, *p2* is the (outer chain mannose) Golgi form of CPY, and *m* is the mature (vacuolar) form of CPY. The large numbers designate the *end* gene affected, e.g., 8 is *end8-1* and 9 is *end9-1*, and *WT* is RH144-3A. The *end12-1* mutant (12) fails to process CPY to the mature form efficiently and secretes the *p2* form. All other mutants process CPY with wild-type kinetics. Notice that the *end13* mutant (13) secretes some *p2* CPY as well, in agreement with the results shown in Fig. 8. The small amount of *p1* and *p2* CPY present in the extracellular fractions from the wild-type strain and the *end9-1* and *end11-1* strains is probably released by occasional lysis of the labeled cells during spheroplasting.

sociated with growth factor receptors which have tyrosine kinase activity and it has been suggested that PI3-kinase may be required both for signal transduction from these receptors and perhaps for their internalization (Panayotou and Waterfield, 1992). In this respect it is interesting that we see no defect in internalization of α -factor in the *end12-1* and *end12-2* mutants. The defect in endocytosis lies at a later step in the pathway.

There are four possible explanations for the defects in the *vps34* mutant. (1) It is possible that Vps34p is required for budding or transport of vesicles from an endosome/prevacuolar compartment to the vacuole. This step is probably required for both vacuolar hydrolase delivery and for delivery of endocytosed material to the vacuole. (2) Vps34p may principally be involved in targeting of proteins from the Golgi apparatus to endosomes. In this case, a protein that is essential for transport of endocytic material from endosomes towards the vacuole would not be delivered to its site of action, and an indirect block of endocytosis would ensue. (3) Vps34p could be directly required for the recycling of essential Golgi components from endosomes back to the Golgi. This could cause a depletion of the vacuolar protein sorting machinery in the Golgi, and an accumulation of this machinery in endosomes, thus possibly impairing endosomal function. (4) Vps34p could be working independently in the two pathways, controlling the sorting of vacuolar hydrolases in the Golgi, and the sorting of proteins in endosomes for traffic to different destinations including the plasma membrane, Golgi, and the vacuole. Experiments using a *vps34* mutant that is temperature-sensitive for function may be valuable in distinguishing between these possibilities.

The *end12/vps34* mutations have much stronger effects on maturation of vacuolar hydrolases than the *end13-1* mutation, and it may be that the effect of *end13-1* on delivery of vacuolar hydrolases is not direct. For example, the *end13-1* mutation may not affect the compartments through which vacuolar hydrolases pass, but a communicating compartment. A defect in the latter compartment may then have a delayed effect on the function of the former compartments and lead to mis-sorting of vacuolar hydrolases.

The uptake-defective *end* mutants can be divided into two classes. One class contains *end8-1*, *end9-1*, *end3*, and *end4*. This class is affected in initial and subsequent uptake kinetics. A second class of mutant contains the *end10-1* and *end11-1* mutants. These are affected in uptake of receptor-ligand complexes also, but the defect does not occur immediately after addition of ligand. These mutants show nearly normal initial α -factor uptake kinetics, but uptake apparently reaches a plateau after 5–10 min of internalization. The first class of mutant is probably directly affected in uptake of α -factor. Several explanations could account for the phenotype of the second class of mutant. (a) A component necessary for uptake of α -factor could become depleted shortly after internalization commences. This explanation is unlikely, since fluid-phase endocytosis is also affected in these mutants and it occurs constitutively. (b) A block in endocytic transport to the vacuole from certain intermediate organelles (e.g., transfer from early to late endosomes) might lead to recycling of receptor-ligand complexes from these intermediates back to the surface, thus producing a new equilibrium with approximately equal uptake and recycling rates. In support of this proposal, the small amount of

internalized α -factor appears not to be degraded with wild-type kinetics in the *end11-1* mutant (data not shown). Further experiments are currently underway to directly address this question.

Interestingly, all but one of the mutations isolated on the basis of their Vsl^- phenotype segregated in crosses with a tightly linked temperature-sensitive growth phenotype. This Ts^- phenotype was most pronounced in *end8-1*, *end9-1*, *end10-1*, *end12-1*, and *end12-2*, and the cells were unable to grow at all at 37°C. The growth of *end13-1* cells was partially impaired at 37°C, and was completely blocked at 39°C. This result extends our previous observation that *end3* and *end4* mutants were Ts^- for growth and supports our speculation that at least one step of the endocytic pathway is essential for growth at elevated temperature. Of the new mutants, only *end11-1* was not strongly defective in growth at high temperature. It remains to be determined whether the defect in *end11-1* is partial, or whether *END11* is not essential for growth at elevated temperatures. The *yp17* mutant is not Ts^- for growth, even though it has a strong defect in endocytosis (Wichmann et al., 1992; Schimmöller and Riezman, 1993). This protein seems to act at one of the latest stages of the endocytic pathway: transport out of the late endosome. It could be that late steps are not required for growth at elevated temperature or that the function required for growth at high temperature is provided by another protein in *yp17* mutant strains.

The Ts^- growth defect exhibited by the new *end* mutants will allow rapid cloning of the wild-type genes by complementation. Further characterization of the mutants described here and a molecular analysis of the corresponding genes will lead to a greater understanding of the cellular activities and structures required for endocytosis in yeast.

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