

end3 and *end4*: Two Mutants Defective in Receptor-mediated and Fluid-phase Endocytosis in *Saccharomyces cerevisiae*

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Abstract. α -factor, one of two peptide hormones responsible for synchronized mating between *MATa* and *MAT α* -cell types in *Saccharomyces cerevisiae*, binds to its cell surface receptor and is internalized in a time-, temperature-, and energy-dependent manner (Chvatchko, Y., I. Howald, and H. Riezman. 1986. *Cell*. 46:355–364). After internalization, α -factor is delivered to the vacuole via vesicular intermediates and degraded there consistent with an endocytic mechanism (Singer, B., and H. Riezman. 1990. *J. Cell Biol.* 110:1911–1922; Chvatchko, Y., I. Howald, and H. Riezman. 1986. *Cell*. 46:355–364). We have isolated two mutants that are defective in the internalization process. Both mutations confer a recessive, temperature-sensitive growth phenotype upon cells that

co-segregate with their endocytosis defect. Lucifer yellow, a marker for fluid-phase endocytosis, shows accumulation characteristics in the mutants that are similar to the uptake characteristics of ^{35}S - α -factor. The endocytic defect in *end4* cells appears immediately upon shift to restrictive temperature and is reversible at permissive temperature if new protein synthesis is allowed. Furthermore, the *end4* mutation only affects α -factor internalization and not the later delivery of α -factor to the vacuole. Other vesicle-mediated processes seem to be normal in *end3* and *end4* mutants. *END3* and *END4* are the first genes shown to be necessary for the internalization step of receptor-borne and fluid-phase markers in yeast.

ENDOCYTOSIS plays a key role in cell physiology, foremost of which is its function in the uptake of micronutrients such as iron (Hemmaplardh and Morgan, 1976; Karin and Mintz, 1981) and cholesterol (Anderson et al., 1977). Other possible roles for endocytosis include the regulation of hormone response by clearance of responsive elements from the membrane (downregulation; Beguinot et al., 1984), recapture of desialylated proteins and lysosomal proteins from extracellular fluids (Spiess, 1990; Kornfeld and Mellman, 1989), antigen processing and presentation (Harding and Unanue, 1989), transcellular transport of immunoglobulins (Kuhn and Krahenbuhl, 1982), and controlled redistribution of plasma membrane proteins between apical and basolateral surfaces of cells (Bartles et al., 1987; Matter et al., 1990).

As is evident in the examples listed above, most of the studies to date have involved the use of mammalian cells. However, endocytosis has also been described in the budding yeast, *Saccharomyces cerevisiae* (Riezman, 1985). Using Lucifer yellow-CH (LY),¹ a marker for fluid-phase internalization in mammalian cells, we provided the first evidence

that the endocytic pathway exists in yeast. LY was shown to accumulate in the yeast vacuole with nonsaturable kinetics under conditions showing time, temperature, and energy dependence. Furthermore, several of the secretory mutants, namely those conditionally defective in the later steps of secretion were also found to be defective in the accumulation of LY, suggesting some physical or operational overlap between the pathways of secretion and endocytosis in yeast.

Subsequent studies by Chvatchko et al. (1986) showed that α -factor, one of the two peptide pheromones secreted by haploid cell types of *S. cerevisiae* for mating preparation and conjugation, is also internalized, however only when specifically bound to *Mata* cells. Once internalized, α -factor is delivered to the vacuole via a vesicular intermediate where it is rapidly degraded in a vacuolar protease-dependent process (Chvatchko, 1987; Dulic and Riezman, 1989; Singer and Riezman, 1990). Concurrent with these findings, Jenness and Spatrick (1986) showed that cellular uptake of α -factor is accompanied by a loss of α -factor binding sites from the plasma membrane. After this initial period of “down-regulation,” cultures reaccumulate newly synthesized receptor sites at the cell surface. These two studies provided corroborative evidence for receptor-mediated endocytosis in yeast and in conjunction with the LY results opened the problem to genetic and biochemical dissection using this simple eukaryote.

In animal cells several different selection techniques have

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1. *Abbreviations used in this paper:* CPY, carboxypeptidase Y; EMS, ethylmethanesulfonate; LY, Lucifer yellow; ts, temperature sensitive.

been developed intending to isolate mutants specifically blocked in the endocytic pathway (Colbaugh et al., 1988; Robbins et al., 1983; Krieger et al., 1985; Merion et al., 1983). However, the mutants isolated to date are not specific to this pathway and also show defects in organelle acidification and/or secretion. In addition, even though many polypeptides, including clathrin, its associated proteins (Pearse and Robinson, 1990), and small GTP binding proteins (Chavrier et al., 1990) have been found to be associated with this pathway none of these have been shown to be essential. However, in *Drosophila*, the *shibire* mutant has been shown to be defective in endocytic uptake (Kosaka and Ikeda, 1983). The *shibire* gene encodes the protein dynamin that could possibly act as a microtubule-based motor (Chen et al., 1991; Obar et al., 1990). The precise role of dynamin in endocytosis has not been discovered nor are we aware of studies analyzing constitutive secretion and lysosome biogenesis in the *shibire* mutant.

In yeast, initial attempts to obtain endocytosis mutants using LY accumulation as a screen led to the isolation of two temperature-sensitive mutants, *end1* and *end2* (Chvatchko et al., 1986). Further characterization of the *end1* mutant (Dulic and Riezman, 1989, 1990) has shown that it also affects vacuole biogenesis. As no distinguishable vacuole is present in the mutant it is not possible to say whether it is primarily defective in endocytosis, vacuole biogenesis, or both. Recent results with *end2* have shown that this mutant internalizes α -factor normally, but is defective in its subsequent delivery to the vacuole (D. Hamburger and H. Riezman, personal communication).

In an attempt to identify proteins required for the internalization step in yeast, we screened cells from an ethylmethanesulfonate (EMS)-mutagenized bank for strains defective in ^{35}S - α -factor uptake. In this report, we describe the isolation and phenotypic characterization of two new endocytic mutants identified from this screen, *end3* and *end4*. These mutants are temperature sensitive for growth, fail to internalize α -factor, its receptor, or to accumulate LY in the vacuole at restrictive temperature. Further analysis indicates that secretory traffic and vacuole biogenesis are normal in these mutants and that the *end4* mutation specifically affects the step(s) of the endocytic pathway before the formation of the previously identified endosomal compartments. These mutants represent the first yeast mutants that specifically block the endocytic pathway at the internalization step.

Materials and Methods

Strains, Media, and Reagents

The strains of *S. cerevisiae* used in these experiments were RH144-3D (*MAT α his4 leu2 ura3 bar1-1*), RH144-3B (*MAT α his4 leu2 ura3 bar1-1 end1::LEU2*), RH266-1D (*MAT α end3 ura3 leu2 his4 bar1-1*), RH268-1C (*MAT α end4 ura3 leu2 his4 bar1-1*), and RH1765 (*MAT α ura3 his4 leu2 trp1::URA3 bar1-1*). RH449 (*Mata his4 leu2 ura3 lys2 bar1-1*) transformed with pDA6300 (a 2u-based plasmid containing the *MF α* , *STE13*, and *LEU2* genes, provided by D. Barnes and J. Thorner, University of California, Berkeley, CA) was used for ^{35}S - α -factor production as described previously (Dulic and Riezman, 1989; Dulic et al., 1990). All yeast strains were grown in complete medium (YPUAD) containing 1% yeast extract, 2% peptone, 40 $\mu\text{g}/\text{ml}$ uracil and adenine, and 2% glucose or minimal medium (SD) as described previously (Dulic et al., 1990). Nutritional supplements were added when needed as described (Sherman et al., 1983). Cells used

for radiolabeling and carboxypeptidase Y (CPY) immunoprecipitation were grown in SD low-sulfate medium where ammonium sulfate was at 50 μM , and radiolabeled in SD no-sulfate medium in which all sulfate salts were substituted by chloride salts (Dulic et al., 1990) and containing 2 mg/ml BSA. Inhibitor medium was YPUAD with the addition of 10 mM sodium azide, 10 mM potassium fluoride and 10 mM *p*-tosyl-L-argininemethylester (TAME; Jenness et al., 1983; Jenness and Spatrick, 1986). Carrier-free $\text{H}_2^{35}\text{SO}_4$ was obtained from New England Nuclear (Boston, MA). Biosynthetically labeled ^{35}S - α -factor was purified according to published procedures (Jenness et al., 1983; Dulic et al., 1990). Chemicals used for SDS-PAGE were purchased from Bio-Rad Laboratories (Richmond, CA). LY Carbohydrazide was obtained from Fluka (Buchs, Switzerland). α -factor was synthesized at the Biochemistry Institute of the University of Lausanne (Switzerland).

Mutant Isolation and Screening

Yeast strain RH144-3D was mutagenized with EMS (Sigma Chemical Co., St. Louis, MO) as described previously by Novick and Schekman (1979). RH144-3D cells were grown overnight at 24°C in YPUAD to stationary phase. After harvesting by centrifugation, the cells were prepared for mutagenesis by resuspension to 1.4×10^8 cells/ml in 100 mM potassium phosphate buffer, pH 7, containing EMS (final concentration 3%). After 30 min of incubation at 30°C on a rotary shaker the mutagen was quenched by the addition of sodium thiosulfate to a final concentration of 5%. The cells were then washed two times with 5% sodium thiosulfate and finally resuspended in YPUAD (1×10^7 cells/ml split into 10 different tubes) to allow for recovery by incubation at 24°C. After 24 h of recovery, aliquots of each of the 10 separate cultures were plated to YPUAD for single colony isolation. After 2 d of growth at 24°C the colonies were replica plated onto two YPUAD plates (one incubated at 24°C and the other at 37°C) and thermosensitive strains were identified by plate comparison. Clones that grew at 24°C but not at 37°C were subjected to the following screen for α -factor internalization:

Cultures of thermosensitive clones were grown to a density of 1×10^7 cells/ml at 24°C in YPUAD. Once harvested the cells were resuspended to a density of 1×10^9 cells/ml in YPUAD and preincubated 10 min at 24°C or 37°C in a shaking water bath. ^{35}S - α -factor (1×10^5 cpm/ 10^9 cells, specific activity 5–10 Ci/mmol) was then added to both tubes and the incubation continued at each of the respective temperatures for 30 min. Binding and internalization were evaluated by transferring 100- μl samples in duplicate to 20 ml of ice-cold 100 mM potassium phosphate buffer, pH 6, and 20 ml of ice-cold 50 mM sodium citrate buffer, pH 1. The pH 6 samples were filtered immediately through nitrocellulose filters (type HA, 45 μm , Millipore Corporation, Bedford, MA) and subsequently washed with 15 ml of the same buffer. pH 1 samples were allowed to sit on ice for 20 min before filtering and washing as described above. All filters were baked at 90°C for 1 h before adding 5 ml of Emulsifier (Packard, Groningen, Netherlands) safe for scintillation counting. Strains which bound ^{35}S - α -factor at wild-type levels but failed to internalize it were next screened for protein synthesis ability (Chvatchko et al., 1986). Colonies were grown in minimal medium containing 200 μM ammonium sulfate at 24°C overnight. 2×10^7 cells were harvested and resuspended in 40 μl of no-sulfate minimal medium and allowed to preincubate 10 min at either 24 or 37°C. 25 μl of SD no-sulfate medium containing 3 μCi $\text{H}_2^{35}\text{SO}_4$ was then added and the incorporation of $^{35}\text{SO}_4^{2-}$ checked at 5, 10, and 30 min by withdrawing 15- μl samples and diluting them into 500 μl 10% ice-cold TCA. After 30 min on ice these samples were filtered, washed with 10% TCA, ethanol, ethanol:ether (50/50, vol/vol), and ether before counting in Emulsifier-safe scintillation fluid. Mutants that failed to incorporate ^{35}S into TCA-precipitable counts were discarded. Two mutants, RH1565 (*MAT α end3 ura3 his4 leu2 bar1-1*) and RH1574 (*MAT α end4 ura3 his4 leu2 bar1-1*) were found in the first 113 temperature-sensitive clones screened. Before experimentation with these mutants was continued, they were backcrossed twice to RH1765, a strain that is isogenic to the parent, RH144-3D and the tetrads were dissected and analyzed using standard genetic techniques (Sherman et al., 1983). Two segregants from the second backcross, RH266-1D(*end3*) and RH268-1C(*end4*), were used for all experiments presented in this paper.

α -factor Internalization and Receptor Clearance Assays

Internalization of α -factor was assayed as described (Dulic et al., 1990) using biosynthetically labeled ^{35}S - α -factor.

Downregulation of receptor sites was assayed in *end3*, *end4*, and wild-type strains essentially as described (Jenness and Spatrick, 1986). In brief, strains were grown overnight in YPUAD medium at 24°C to a density of 1×10^7 cells/ml. After harvesting, two 120-ml cultures containing 5×10^6 cells/ml in YPUAD and 10 mM TAME were prepared. The first stage of the experiment (receptor clearance) was initiated with the addition of α -factor (10^{-7} M final concentration) to one of the cultures and the same volume of water to the other culture as a control. Incubation was continued at either 24 or 35°C and at the indicated times 20-ml aliquots (1×10^8 cells) were withdrawn and collected on nitrocellulose filters. Filters containing cells were washed two times with 20 ml of inhibitor medium (see Strains and Media section for composition) and the cells were subsequently resuspended in 20 ml of inhibitor medium at 25°C to allow for dissociation. After 2 h of incubation the cells were harvested and resuspended in 500 μ l of ice-cold inhibitor medium and held on ice for up to 2 h before beginning Stage II of the experiment. To determine the number of α -factor binding sites remaining after each time of preexposure to α -factor in Stage I, two duplicate binding reactions were set up for Stage II of the experiment as follows. In this procedure, ^{35}S - α -factor (30,000 cpm) was added to 100 μ l of cells (2×10^7 cells) in inhibitor medium for the test reactions and ^{35}S -labeled α -factor (30,000 cpm), 2×10^7 cells and unlabeled α -factor to a final concentration of 2×10^{-5} M were combined for the control reactions. After an incubation of 30 min at 25°C, 90 μ l (representing 1.5×10^7 cells) were diluted into 20 ml of inhibitor medium and filtered using Whatman GF/C filters (Millipore Corp.) Filters were washed once with 20 ml of inhibitor medium before being dried for scintillation counting in 5 ml Emulsifier-Safe scintillant. Those counts which could be competed by 2×10^{-5} M unlabeled α -factor were considered to represent specific binding.

LYCH Accumulation

LY accumulation experiments were performed as described (Riezman, 1985) with the following minor changes. Yeast cultures grown overnight at 24°C were harvested and resuspended to 10^7 cells/ml for the assay. One aliquot of cells were slowly brought to 35°C by adjusting the temperature setting on the water bath from 24 to 35°C. Once 35°C was reached, LY was added to 4 mg/ml. To the other aliquot LY was immediately added to 4 mg/ml. After 2 h at 24°C or 1 h at 35°C, the cells were prepared for microscopic viewing by washing extensively with ice-cold 50 mM sodium succinate buffer, pH 5, containing 10 mM sodium azide. The washed cells were then applied to poly-L-lysine-coated microscopic slides. Cells were observed using a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) and fluorescence or Nomarski optics as described (Riezman, 1985). Photographs were made using Kodak, T-Max 400 film (Eastman Kodak Co., Rochester, NY) pushed to ASA 1,600 for the fluorescence pictures. Exposures were 20 to 25 s. To quantify the results cells with a clear vacuolar staining with LY were counted as positive. Cells without vacuolar staining, but with a clearly distinguishable vacuole by Nomarski optics were counted as negative to ensure that the vacuole was in focus. A minimum of 46 cells were counted.

Invertase Secretion Assay

Yeast cells were grown overnight at 24°C in YPUAD medium containing 5% glucose to approximately 8×10^6 cells/ml. The culture was shifted to 37°C slowly by simply readjusting the temperature setting on the waterbath from 24 to 37°C. 10 min after the waterbath reached 37°C, 3.5×10^7 cells were removed and put on ice as an uninduced control. The remaining cells were harvested by centrifugation, washed two times with water equilibrated to 37°C and resuspended to the original volume (8×10^6 cells/ml) in prewarmed (37°C) YPUAD containing only 0.1% glucose. At the indicated times, 3.5×10^7 cells were removed, pelleted at 4°C, and resuspended in 500 μ l ice-cold 10 mM sodium azide. From each 500 μ l aliquot, 250 μ l were removed for the determination of the amount of external invertase present and the other 250 μ l used for the determination of the total (internal + external) invertase activity present. The amount of total invertase activity was determined by pelleting the cells taken at each time point and resuspending them in the original volume of ice-cold 1% Triton X-100, 10 mM sodium azide. After freezing and thawing the aliquots once in liquid nitrogen, the cells were pelleted and washed again with cold 10 mM sodium azide and finally resuspended in 250 μ l 10 mM sodium azide. The amount of external invertase present was determined directly using the other half of the aliquot taken at each time point. Invertase assays were performed as described by Goldstein and Lampen (1975) using incubations without sucrose as controls.

CPY Biogenesis

Radiolabeling of cells, either *end3* (RH266-1D), *end4* (RH268-1C), *end1* (RH144-3B), or wild type (RH144-3D) for immunoprecipitation of CPY was accomplished using a method similar to that published by Dulic and Riezman (1989). Strains were first grown to stationary phase in YPUAD medium. The cells were harvested, washed in SD low-sulfate medium and used to inoculate a 50-ml culture of SD low-sulfate medium to a density of 5×10^6 cells/ml. This culture was then allowed to incubate 6 h at 24°C to induce the sulfate permease. The low sulfate culture was then harvested and the cells washed with SD no-sulfate media and finally resuspended to 2×10^8 cells/ml in SD no-sulfate medium containing 2 mg/ml BSA. Cells (2.5 ml) were preincubated 10 min at 37°C before adding 25 μ l of $\text{H}_2^{35}\text{SO}_4$ (40 mCi/ml). The cells were incubated at 37°C for 5 min. Ammonium sulfate was added to 2 mM final concentration and methionine and cysteine were added each to 20 μ g/ml to initiate the period of chase. At 0, 5, 10, 15, and 30 min of chase, 500 μ l of the culture was removed to microfuge tubes on ice containing 50 μ l of 0.2 M $\text{NaN}_3/0.2$ M NaF. At the end of the chase, intracellular and extracellular (medium plus periplasm) extracts were prepared for immunoprecipitation (Stevens et al., 1986). Briefly, the cells were collected by centrifugation in a microfuge (3,000 g, 5 min) and the supernatant was withdrawn. The cells were converted to spheroplasts using lyticase (Stevens et al., 1986) for 45 min at 30°C in the presence of 20 mM each NaN_3 and NaF. The spheroplasts were collected (3,000 g, 5 min) and the supernatant (periplasm) removed to a fresh tube. The medium and periplasm were denatured by adding SDS to 1%, boiled immediately, and then combined. The spheroplasts were resuspended in 95°C 1% SDS (0.5 ml) and immediately heated at 95°C for 3–5 min. The extracts were then clarified in the microfuge at 15,000 g for 30 min. Sodium azide was added to 10 mM and the immunoprecipitations were performed as described (Gasser et al., 1982) using a polyclonal antibody raised in rabbits against CPY. Immunoprecipitates were analyzed by SDS-PAGE (Laemmli, 1970) on 10% gels with fluorographic enhancement using 1 M sodium salicylate. The films were quantified by scanning using a densitometer (Molecular Dynamics, Sunnyvale, CA).

α -factor Degradation Assay

α -factor degradation assays were performed as described by Dulic and Riezman (1989). *end4* (RH268-1C) and wild-type cells (RH144-3D) grown at 24°C to $<10^7$ cells/ml were harvested and allowed to bind ^{35}S - α -factor (20,000 cpm/ 10^9 cells) on ice for 1 h. 100- μ l duplicate aliquots were removed to (a) determine the total amount of cell-associated radioactivity and the internalized amount of ^{35}S - α -factor by the internalization assay and (b) evaluate the condition of the ^{35}S - α -factor, if internalized, as in a normal degradation assay. Once the aliquots were withdrawn, the remainder of the binding reaction was centrifuged for 3 min at 3,500 rpm at 4°C and the cells resuspended to the same density (1×10^9 cells/ml) in medium prewarmed to 15°C for 20 min to allow α -factor internalization but retard delivery to the vacuole. At 20 min, the cells were pelleted again for 3 min at 3,500 rpm, 4°C and resuspended to the same density (1×10^9 cells/ml) in medium prewarmed to 34°C. At the indicated times after shift to 34°C, 100- μ l aliquots were withdrawn and the α -factor extracted for thin-layer chromatography as described earlier (Dulic and Riezman, 1989). Intact and degraded pheromone were resolved using preparative silica gel 60 plates (2.2-mm-thick, 20 \times 20 cm, Merck, Germany) and the n-butanol:propionic acid:water, 50:25:35 (vol/vol/vol) solvent system used previously (Dulic et al., 1990). EN³HANCE spray (DuPont de Nemours International, S.A., Regensdorf, Switzerland) was used to coat the plates for fluorography using Kodak XAR-5 film (Eastman Kodak Co.).

α -factor Sensitivity

RH144-3D, RH266-1D, or RH268-1C cells were grown to early logarithmic phase and counted. The cells (5×10^5 /ml = $1 \times$ final concentration) were washed with YPUAD and then mixed with YPUAD medium in 0.8% agar at 47°C. 5 ml was immediately poured into petri dishes (94 \times 16 mm; Greiner Labortechnik, Nürtingen, Germany). As soon as the medium solidified, the plates were moved to 4°C until use. α -factor solutions in water were used to apply 10, 20, or 50 ng (in 20 μ l) of α -factor to one side of 6.3-mm filter disks (Schleicher and Schuell, Feldbach, Switzerland) which were then placed, α -factor-side down, on top of the test plates. The plates were then incubated 1–2 d at 24 or 30°C and the resulting halo diameters were measured. Each experiment was performed in duplicate and the halo diameters were averaged.

Results

Isolation and Preliminary Characterization of the *end3* and *end4* Mutants

The primary objective of the initial stages of this work was to begin a molecular dissection of the endocytic pathway for receptor-borne markers in yeast through the isolation and characterization of endocytic mutants. Towards this goal, we used the simple assay of α -factor internalization to screen a bank of temperature-sensitive lethal strains generated using EMS mutagenesis. From a total of 2,000 colonies plated after mutagenesis, 75 mutants temperature sensitive (ts) for growth were isolated. Of these 75 ts mutants, two isolates, later named *end3* and *end4*, were identified in the preliminary screening by determining that <20% of the ^{35}S - α -factor bound at 37°C could be internalized after a 30 min incubation at that temperature. Control incubations of cells with ^{35}S - α -factor at 24°C revealed that one mutant, *end3*, was also unable to internalize α -factor at 24°C, permissive growth temperature. The other mutant, *end4*, internalized ~70% of the total bound α -factor after 30 min at 24°C in control incubations during the initial screen.

Once protein synthesis at 37°C for the *end3* and *end4* mutants was confirmed, inheritance of the temperature sensitive phenotype and the endocytic defect was followed through two generations for each mutant. All *MATa* progeny were tested for α -factor uptake. The ts growth defect always cosegregated with the endocytic defect indicating that a single genetic lesion is responsible for both phenotypes. Diploid strains made from the mating of both mutants with the wild-type strain (RH144-3D) and diploids made from mating the *end3* mutant with the *end4* mutant are not temperature sensitive for growth. Therefore, both mutations are recessive and complement each other. The endocytic defects of the two mutants are also recessive.

end3 and *end4* Mutants Are Defective in α -factor Internalization, Receptor Downregulation, and LY Accumulation

To determine whether these mutants contain true blocks in the endocytosis of α -factor or only affect the rate at which α -factor is taken up, the internalization of ^{35}S - α -factor was followed over time at both permissive and restrictive growth temperatures in each mutant. For these studies, ^{35}S - α -factor was mixed with *end3*, *end4*, and wild-type cells in YPUAD medium pre-equilibrated to either 24 or 37°C. At various times after ^{35}S - α -factor addition, aliquots were withdrawn and diluted into both pH 6 and pH 1 medium to measure the total amount of cell-associated radioactivity and the amount of internalized α -factor, respectively. The results of these experiments are shown in Fig. 1. α -factor internalization was >95% of maximum in wild-type cells at both 24 and 37°C by 30 min. In contrast, only 16 and 12% of the ^{35}S - α -factor bound to *end3* cells was taken up by 30 min at 24 and 37°C (Fig. 1). This level of internalization did not increase even if incubation times were extended to 90 min at both temperatures for *end3* cells. When *end4* cells were incubated with ^{35}S - α -factor at 24°C, internalization was again virtually complete by 30 min (>90% of maximum), however the amount of bound α -factor internalized by *end4* was less than wild type (72% that of wild type). At restrictive temper-

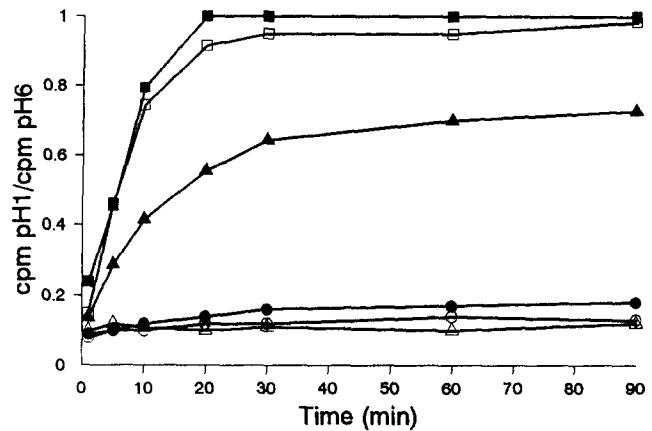


Figure 1. Internalization of ^{35}S - α -factor by *end3*, *end4*, and wild-type cells. *end3* (\circ), *end4* (Δ), or wild-type cells (\square) were preincubated 10 min at either 24 (closed symbols) or 37°C (open symbols) before ^{35}S - α -factor was added and the incubation continued at either 24 or 37°C. At the times indicated, duplicate samples were diluted to either ice-cold 100 mM potassium phosphate buffer, pH 6 or ice-cold 50 mM sodium citrate buffer, pH 1 buffer and filtered to determine the extent of α -factor internalization. The values represent averages from four experiments.

ature *end4* cells, like *end3* cells, internalized very little bound α -factor. α -factor internalization at 37°C by *end4* cells never exceeded 12% of the total bound pheromone even after 90 min of incubation. The installation of the α -factor uptake block in *end4* cells requires no preincubation at nonpermissive temperature ($\geq 34^\circ\text{C}$; data not shown). Using initial rates of uptake calculated from experiments performed on cells with ^{35}S - α -factor prebound at 0°C (pulse-chase protocol; Dulic et al., 1990), the half-times of maximal internalization ($t_{1/2}$) of α -factor by the wild-type strain were 9.4 and 8.4 min at 24 and 37°C, and 15.6 min at 24°C for *end4* cells.

Internalization of α -factor by *MATa* cells has been shown to be accompanied by a coordinate loss of pheromone-occupied cell-surface binding sites (Jenness et al., 1986). To determine whether the mutants clear α -factor receptor binding sites from the cell surface, actively growing cultures of wild type, *end3* and *end4* strains were subjected to the assay for receptor downregulation (Jenness and Spatrick, 1986). In brief, after preincubating the cells 10 min at either 24 or 35°C, cultures were divided such that one half received 1×10^{-7} M unlabeled α -factor and one half received no α -factor at all. At various times, aliquots were withdrawn, the cells washed extensively with YPUAD medium containing the metabolic inhibitors NaN_3 and NaF , diluted and held at 25°C to allow dissociation of surface-bound α -factor. The binding capacity of each cell sample was subsequently determined by incubation with ^{35}S - α -factor and appropriate processing for the measurement of cell-bound radioactivity as explained in Materials and Methods. A nonpermissive temperature of 35°C was chosen for this experiment because there is less α -factor-independent loss of cell surface binding activity than at 37°C. As shown in Fig. 2, upon addition of α -factor at either 24°C or 37°C, the wild-type strain lost between 60 and 65% of its surface binding capacity in the first 15 min (Fig. 2). *End3* cells failed to clear a large proportion of receptor binding sites from the cell surface at both

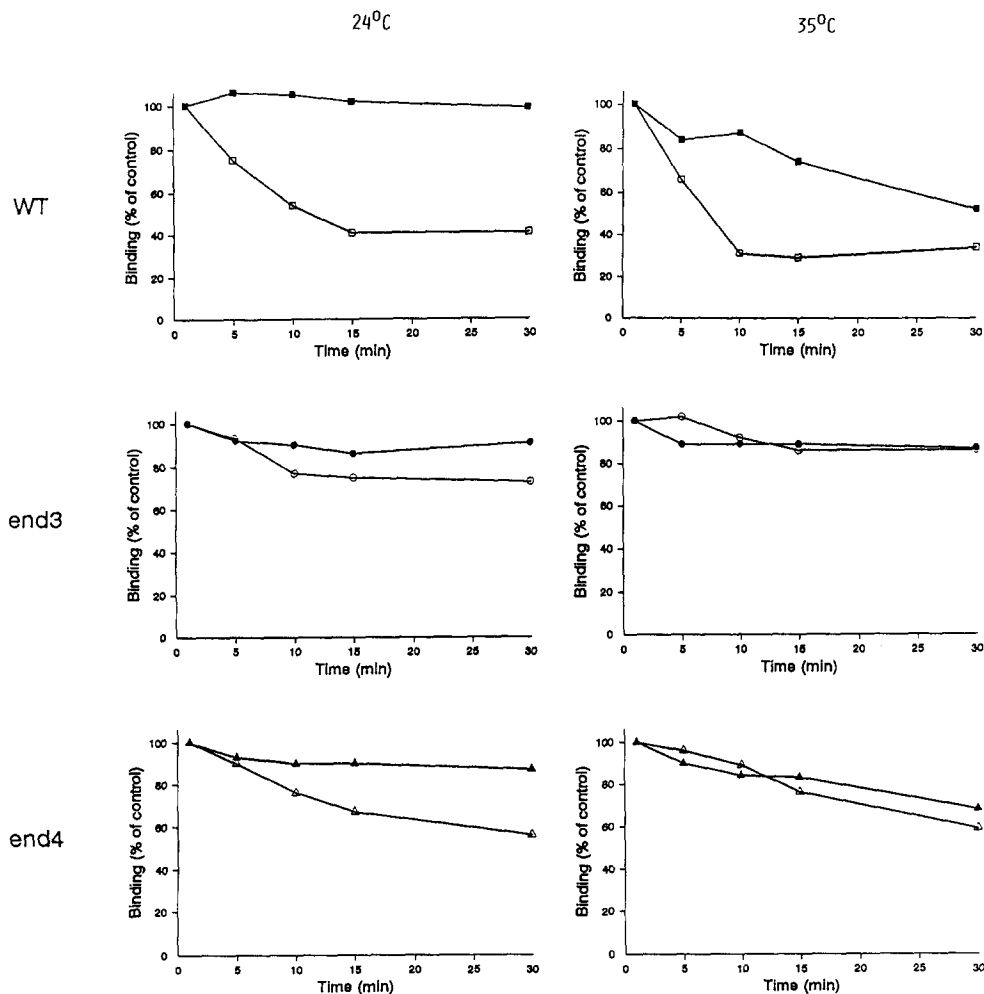


Figure 2. Downregulation of the α -factor receptor in wild type, *end3*, and *end4* cells. Wild type, *end3*, and *end4* cells were preincubated for 10 min at either 24 or 35°C in YPUAD medium containing 10 mM TAME. At time zero one half of each culture received 1×10^{-7} M unlabeled α -factor (open symbols) or no α -factor (closed symbols). Duplicate samples were withdrawn at the times indicated, washed thoroughly with inhibitor medium, and further incubated to allow dissociation of the surface bound α -factor and finally assayed for their ability to bind ^{35}S - α -factor. Values represent the averages from four experiments.

permissive and restrictive temperatures. There was a small α -factor-dependent decrease in cell surface binding activity in *end3* cells. This could have been due to a decrease in receptor binding affinity after α -factor administration due to dissociation from the tripartite G protein (Blumer and Thorner, 1990) or alternatively, could have resulted from an incomplete dissociation of the bound nonradioactive α -factor in the assay. One can also see that cell surface binding activity was particularly stable in the *end3* mutant at 37°C in the presence or absence of α -factor. We have no explanation for this phenomenon. As receptor stability was increased with or without pheromone and only at 37°C we did not pursue it further.

At 24°C *end4* cells showed slightly more α -factor-dependent loss of cell surface binding activity than *end3* cells, but the amount of this loss was significantly less than wild-type cells under identical incubation conditions (Fig. 2). In four separate experiments a similar relationship was found between the different strains even though the magnitude of the α -factor-dependent reduction in cell surface binding activity varied. The results from this set of experiments support the data from Fig. 1. Although both *end3* and *end4* strains are temperature sensitive for growth, only *end4* shows a temperature-sensitive endocytic defect. The relatively small difference found between *end3* and *end4* strains in this assay could reflect the nature of the assay itself (see Discussion). En-

docytosis of α -factor and its receptor are blocked at both 24 and 37°C in the *end3* mutant.

Since the *end3* and *end4* mutants were originally isolated using a screen based on α -factor internalization, we wished to determine whether these two mutations also affect the internalization of the fluid-phase endocytic marker, LY. *end3*, *end4*, and wild-type cells were incubated in LY for a period of 1 h at 35°C or 2 h at 24°C. Aliquots were washed extensively to remove excess LY and the cells were mounted for viewing under Nomarski and fluorescence optics. The results of this experiment are shown in Fig. 3. At 24 and 35°C LY accumulated in the vacuoles of the wild-type strain as expected. *end3* cells, on the other hand, showed almost no accumulation of LY at either 24 or 37°C. The extremely bright fluorescence seen in the *end3* panels (Fig. 3) emanates from lysed cells. LY accumulation in the vacuole of *end4* cells was somewhat temperature sensitive. At 24°C there was a higher background which seems to be due to a higher binding of LY to the cell wall at this temperature in this strain. At 35°C no vacuolar staining of *end4* cells were detected (Fig. 3). As the presence of lysed cells precluded the quantitation of LY internalization as described previously (Riezman, 1985) we quantified the percentage of cells showing clear vacuolar staining. At 24°C, 92% of the wild-type cells showed vacuolar labeling whereas only 4% of the *end3* cells and 10% of the *end4* cells did. At 35°C, 97% of the wild-type cells

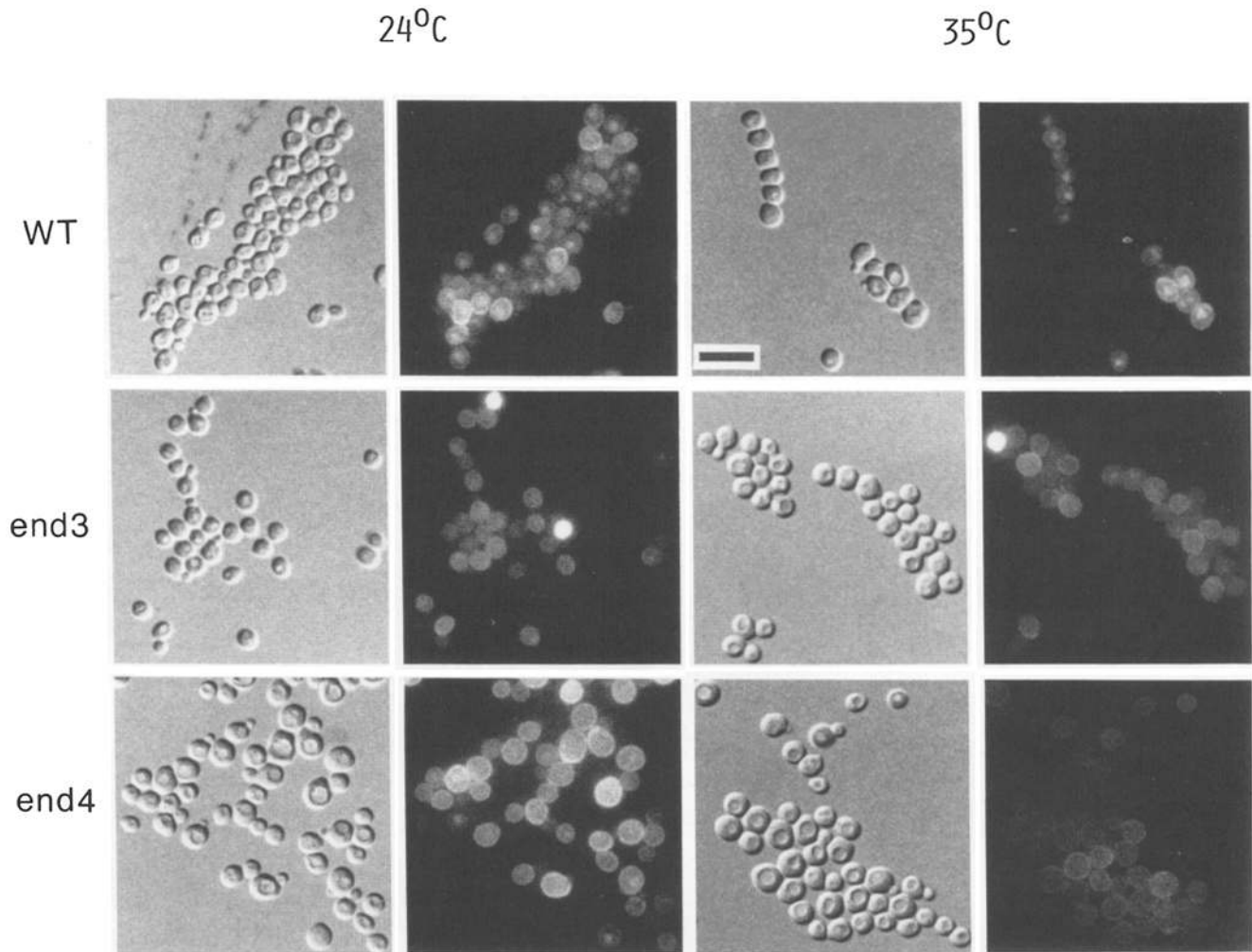


Figure 3. Accumulation of LY-CH by *end3*, *end4*, and wild-type cells. *end3*, *end4*, and wild-type cells were grown overnight at 24°C. After harvest, one half of the culture was allowed to continue incubation at 24°C while the other half was slowly shifted to 35°C. After 35°C was reached, all cells were incubated with 4 mg/ml LY-CH (1 h for 35°C sample, 2 h for 24°C sample), washed and mounted as described in Materials and Methods. Cells were visualized using Nomarski (left columns) and fluorescence optics (right columns). Bar, 10 μ m.

showed vacuolar labeling whereas 4% of *end3* cells and 0% of *end4* cells showed labeling. The LY phenotypes are most consistent with the α -factor receptor clearance phenotypes shown in Fig. 2 for these two mutants (see Discussion). These data clearly show that the genes defined by the *end3* and *end4* mutations are necessary for both receptor-mediated and fluid-phase endocytosis in yeast.

Secretion of Invertase and the Maturation of Carboxypeptidase Y Are Normal in the *end3* and *end4* Mutants

Using LY we (Riezman, 1985) showed that some of the yeast mutants conditionally defective in secretion are also defective in endocytosis. This finding suggested some functional overlap between the protein participants of both processes. To determine whether the *end3* or *end4* mutations interfere with the transit of proteins through the secretory pathway, the transport of two well-characterized markers, invertase and carboxypeptidase Y, was followed at 37°C. After induction of invertase synthesis with a shift from 5 to 0.1% glucose, invertase secretion was followed using the standard coloro-

metric assay developed by Goldstein and Lampen (1975). Fig. 4 shows the results from these experiments. Neither the induction nor the secretion of invertase were affected by incubation of the *end3* or *end4* mutants at restrictive temperature.

CPY, the second marker of secretion followed in this study, is a soluble vacuolar protease. During transport to the vacuole it follows another branch of the pathway from the Golgi complex to the vacuole instead of from the Golgi complex to the plasma membrane. The transit of CPY through the secretory pathway begins in the ER. After cleavage of the signal peptide and core glycosylation a 67-kD form, p1 CPY, is generated that is transported to the Golgi complex where the core oligosaccharides are extended yielding p2 CPY (69 kD). Finally, before or upon arrival of p2 in the vacuole, the amino-terminal prosequence (~8 kD) is removed to yield the mature protease (m CPY) (Stevens et al., 1982). To assess the integrity of this pathway, wild type, *end3* and *end4* cells were pulse-labeled with $H_2^{35}SO_4$ for 5 min at 37°C. The pulse was followed by a period of chase initiated by adding unlabeled ammonium sulphate, methionine, and cysteine also at 37°C. At various times during the chase, aliquots of

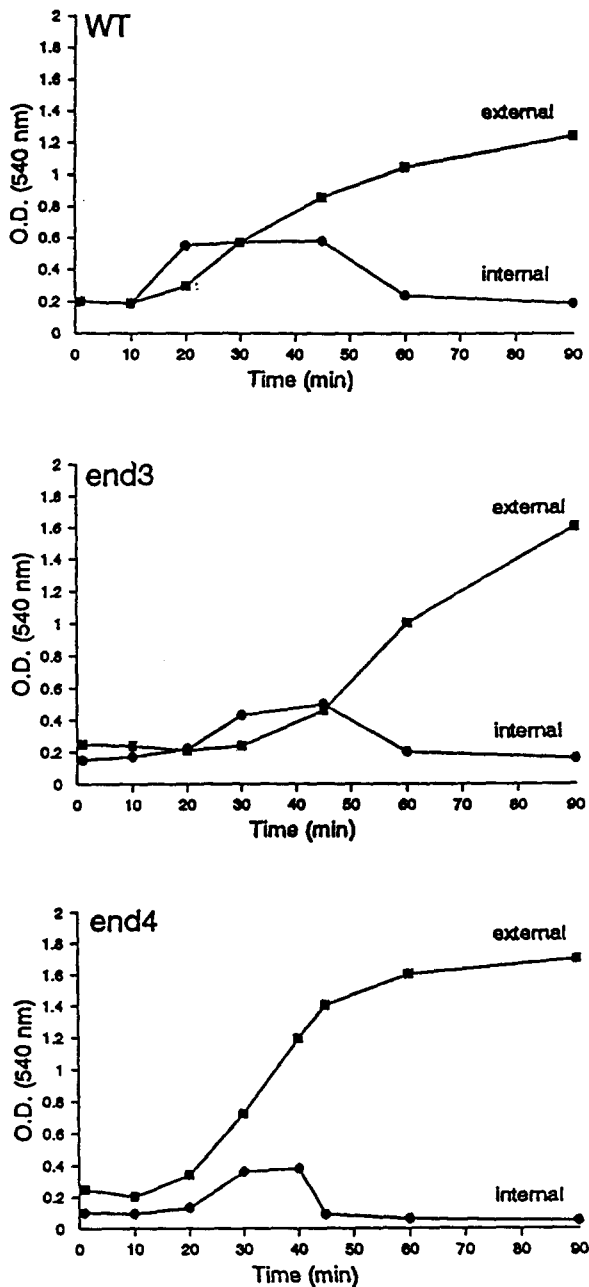


Figure 4. Invertase secretion by *end3*, *end4*, and wild-type cells. *end3*, *end4*, and wild-type cells were grown overnight at 24°C in YPUAD containing 5% glucose. Cultures were then shifted slowly to 37°C. 10 min after 37°C was reached, cells were harvested and resuspended in media containing 0.1% glucose for invertase induction. At the times indicated, aliquots were withdrawn for assay of total and external invertase activity as described in Materials and Methods.

cells were withdrawn, converted to intracellular and extracellular fractions (Stevens et al., 1986), and the CPY was precipitated using an anti-CPY antibody. Fluorographs of the 10% SDS-polyacrylamide gels used to resolve the resulting immunoprecipitates from the intracellular extracts are shown in Fig. 5. The rate of appearance of p1, p2, and mCPY were the same in the three strains. All of the p1 and p2 CPY could be chased to the mature form. As quantified by densitometry a maximum of 13% of the CPY at any time point

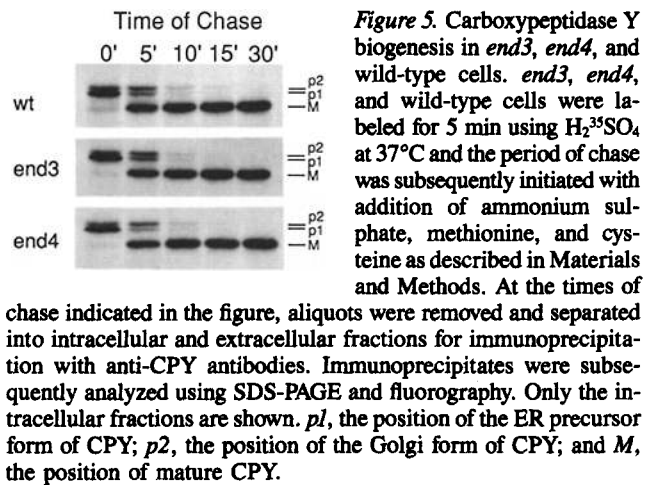


Figure 5. Carboxypeptidase Y biogenesis in *end3*, *end4*, and wild-type cells. *end3*, *end4*, and wild-type cells were labeled for 5 min using $H_2^{35}SO_4$ at 37°C and the period of chase was subsequently initiated with addition of ammonium sulphate, methionine, and cysteine as described in Materials and Methods. At the times of chase indicated in the figure, aliquots were removed and separated into intracellular and extracellular fractions for immunoprecipitation with anti-CPY antibodies. Immunoprecipitates were subsequently analyzed using SDS-PAGE and fluorography. Only the intracellular fractions are shown. p1, the position of the ER precursor form of CPY; p2, the position of the Golgi form of CPY; and M, the position of mature CPY.

The α -factor Internalization Defect in *end4* Is Reversible

was found in the extracellular fractions (data not shown). As a control for a mutant that is defective in vacuolar protein sorting, the *end1* mutant (Dulic and Riezman, 1989), which is in the same complementation group as *vps11* (Rothman et al., 1989), was tested in the same assay. No maturation of CPY was detected in the 30-min chase period and >30% of the p2 CPY was found in the extracellular fraction. The much smaller amounts of CPY found in the external fractions in the wild-type, *end3*, and *end4* cells probably resulted from some lysis during spheroplast preparation because the “external” forms were found irrespective of the time of chase and irrespective of the form (p1, p2, or m) of CPY. In any event, such small amounts of “external” CPY would not be indicative of a defect in vacuolar protein sorting (Robinson et al., 1988; Rothman and Stevens, 1986).

A necessary prerequisite to further characterization of the *end4* mutant was to determine whether the endocytic defect present at restrictive temperature could be reversed upon return of *end4* cells to permissive temperature. For this experiment, the endocytic defect was first installed in *end4* by incubation at 34°C for 10 min. The preincubated cells were then incubated with ^{35}S - α -factor at 34°C for an additional 10 min and then shifted either to 24°C in YPUAD medium or to 24°C YPUAD in medium containing cycloheximide. One culture maintained at 34°C throughout all steps of the experiment provided the control. The results of this experiment are found in Fig. 6. Even after 90 min of incubation at 34°C, *end4* cells internalized only 20% of the ^{35}S - α -factor that was bound. *end4* cells first preincubated at 34°C, then incubated with ^{35}S - α -factor at 34°C before shift to fresh medium at 24°C showed a short, reproducible delay before ultimately internalizing 82% of the total bound α -factor. Addition of cycloheximide to the cells using this same protocol reduced the amount of internalized α -factor after 90 min to the control level. Therefore reversibility of the *end4* endocytic defect requires the synthesis of new protein. Protein synthesis in general, however, is not required for α -factor uptake after a 34°C preincubation because wild-type cells treated with cycloheximide internalized α -factor with kinetics identical to cells which receive no cycloheximide

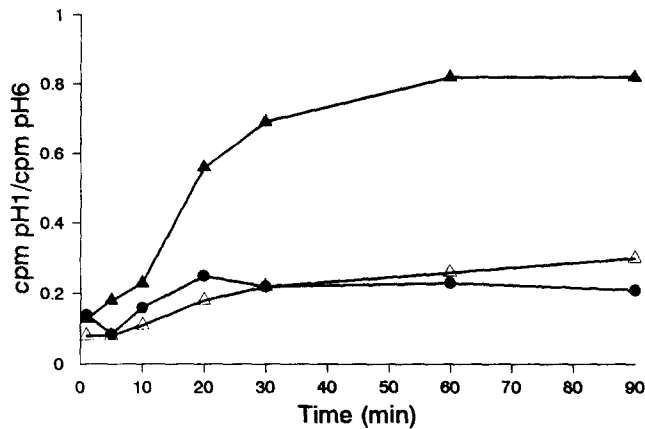


Figure 6. Reversibility of the *end4* mutation. *end4* cells were allowed to preincubate for 10 min at 34°C. ³⁵S- α -factor was added and the incubation continued at 34°C for 10 min. After this 10-min incubation, the cells were pelleted and resuspended in either 24°C medium (▲), 34°C medium (●), or 24°C medium containing cycloheximide (△) (1.4×10^{-4} M). At the time points indicated aliquots were withdrawn for processing at pH 6 and pH 1 as described in Materials and Methods. The values represent averages from two experiments.

(data not shown). This result supports the idea that the inactivation of the *end4* mutant protein at restrictive temperature is irreversible. Only newly synthesized End4 protein can reverse the endocytic defect once installed at restrictive temperature in this mutant.

The *end4* Mutation Affects Only the Earliest Step(s) in Internalization and not the Later Delivery of α -factor to the Vacuole

The earliest step in the yeast endocytic pathway that can be monitored currently is the conversion of ³⁵S- α -factor from pH 1 wash sensitivity to pH 1 wash insensitivity. Once α -factor is internalized it is delivered to the vacuole where it is subsequently degraded. This last step in the endocytic pathway can be assessed by extraction of internalized ³⁵S- α -factor and resolution of the degradation products from intact α -factor using a thin layer chromatography system (Chvatchko et al., 1986). Yet another step in the pathway has been defined by Singer and Riezman (1990). Using conditions similar to those found previously in mammalian cells to arrest delivery of endocytosed material to lysosomes, they could detect α -factor in an intermediate compartment(s) between the cell membrane and the vacuole. Their data showed that when *MATa* cells were incubated with ³⁵S- α -factor at 15°C for 20 min, intact α -factor was recovered as vesicular intermediates en route to the vacuole from the plasma membrane. Using this information, we reasoned that if the *end4* protein was involved in any of the steps past these 15°C intermediates, degradation would be blocked in cells allowed first to accumulate α -factor in the 15°C compartment(s) before shifting to the restrictive temperature. Conversely, α -factor should be degraded in *end4* cells if the mutant block precedes the transit of α -factor through the 15°C compartment(s) using this same experimental protocol. Fig. 7 shows the results of an experiment in which the *end4* mutant and wild-type cells were first allowed to bind ³⁵S- α -factor for 1 h on ice, then shifted to medium at 15°C for 20 min to al-

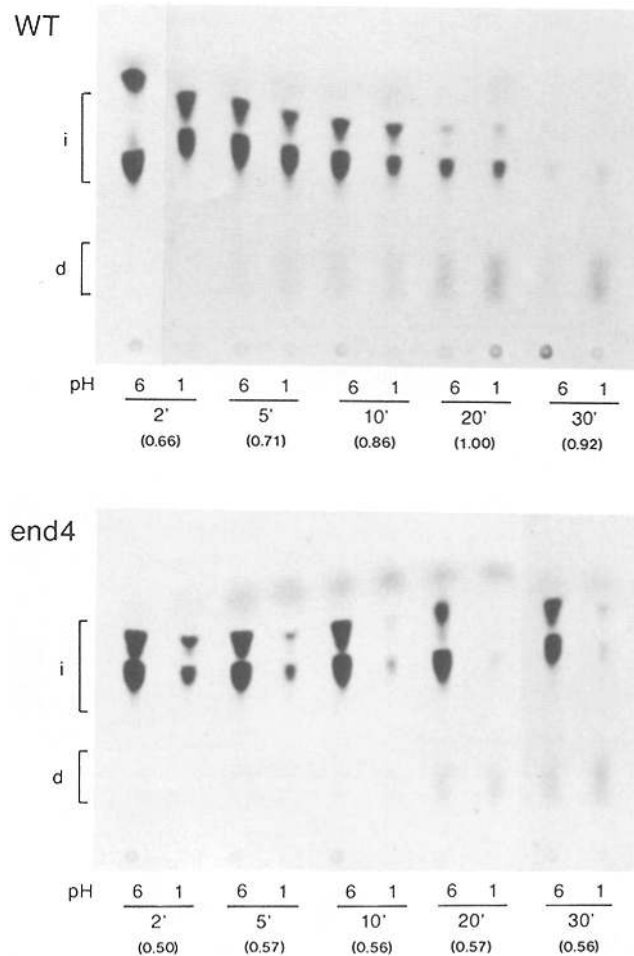


Figure 7. Degradation of α -factor by *end4* and wild-type cells at 37°C after accumulation of α -factor in the intermediate compartment at 15°C. *end4* and wild-type cells were allowed to bind ³⁵S- α -factor for 1 h on ice. The cells were pelleted at 4°C, resuspended in 15°C medium, and incubated for 20 min more at 15°C. At 20 min, the cells were pelleted again at 4°C and resuspended in medium pre-equilibrated to 34°C. At the times indicated after 34°C shift, aliquots were taken and diluted into pH 6 and pH 1 buffer as described for the measurement of internalization as in Fig. 1. α -factor degradation (indicating delivery to the vacuole) was assessed by extracting the cell-associated radioactivity (see Materials and Methods) and developing these samples on preparative TLC plates for subsequent fluorography. The positions of intact α -factor (*i*) and the degradation products (*d*) are noted. Values in parentheses below each pair of lanes indicate the pH 1/pH 6 ratio of duplicate samples taken and processed only for measurement of internalization.

low movement of the bound α -factor into the 15°C compartment(s), before again shifting to 34°C to install the *end4* mutant block. Other experiments have shown that inactivation of *end4* at 34°C is immediate and therefore no preincubation at the restrictive temperature is required to obtain complete inhibition of endocytosis in this mutant (data not shown; see Fig. 7). Aliquots throughout the 34°C incubation were taken for extraction of ³⁵S- α -factor and subsequent analysis of degradation by resolution on thin layer chromatography plates. As can be seen from the quantitation of internalization in this experiment (Fig. 7) internalization of ³⁵S- α -

factor was arrested immediately upon shift to 34°C in the *end4* mutant, but continued during the 34°C chase in the wild-type strain. The ³⁵S- α -factor that was allowed to bind and then accumulate in the 15°C intermediate compartment(s) in the *end4* mutant (pH 1 resistant) was degraded with kinetics identical to wild type when shifted to 34°C. The intact ³⁵S- α -factor that remained associated with *end4* cells at pH 6 was bound to the cell surface and not internalized because it was dissociated by the pH 1 treatment. From this experiment we conclude that the mutation in *end4* affects only the step(s) before or including the formation of the 15°C intermediate compartment and not steps involved in the delivery of α -factor from the intermediate compartment(s) to the vacuole.

Endocytosis Is Not Necessary for Pheromone Response

When α -factor binds to its receptor it induces a signal and is rapidly internalized. It has been shown that the receptor need not be internalized to transmit the pheromone signal (Reneke et al., 1988). It still remains possible that endocytosis of other proteins could play a role in the response. This is now easily testable with the availability of endocytic mutants. To test pheromone sensitivity the "halo" assay was used. In this assay *MATa* cells are embedded in solid growth medium and disks containing α -factor are placed on top of the agar. The radius of the zone of growth inhibition is logarithmically proportional to the sensitivity of the strain. As can be seen in Table I, *end3* cells needed approximately two times less α -factor than *end4* or wild-type cells to form the same size halo at 24°C. At 30°C, both *end3* and *end4* cells were ~ 2.5 times more sensitive to α -factor than wild-type cells. This correlates well with the abilities of the strains to internalize α -factor. At 30°C *end4* cells are still able to grow, but show an almost complete block in α -factor uptake (data not shown). As this difference in sensitivity is small and both *end3* and *end4* strains grow more slowly than wild type, we wondered whether small differences in apparent sensitivity could also be explained by the reduced number of cells. Therefore, we raised and lowered the number of cells embedded in the agar by a factor of 2. As can be seen in Table I, when more cells were embedded in the agar the strain appeared less sensitive to α -factor by this test. This difference was less than the effect seen in the *end* mutants, but indicates that the apparent supersensitivity seen in the mutants may be less than was measured. However, it can be concluded from these experiments that the *end* mutants are capable of responding to α -factor and therefore endocytosis is not necessary for pheromone signaling.

Discussion

By screening an EMS-mutagenized bank for cells unable to internalize α -factor, we have isolated two new yeast endocytic mutants, *end3* and *end4*. Both mutants exhibit thermosensitive defects in growth and although both mutants bind α -factor at levels comparable to wild-type cells, neither mutant internalizes α -factor or its receptor with wild-type characteristics. The *end3* mutant does not internalize α -factor at the permissive growth temperature of 24°C or the restrictive growth temperature of 37°C (Fig. 1). *End4* mutants take up α -factor at permissive temperature, but not at tem-

Table I. α -factor Induced Halo Radii (cm)

Strain and Temperature	°C	Quantity of α -factor		
		10 ng	20 ng	50 ng
Wild type	24	1.35	1.75	2.20
Wild type	30	1.05	1.50	2.00
<i>end3</i>	24	1.70	2.08	2.55
<i>end3</i>	30	1.70	2.10	2.55
<i>end4</i>	24	1.30	1.70	2.20
<i>end4</i>	30	1.68	1.98	2.48
1/2 \times Wild type	24	1.32	1.85	2.62
Wild type	24	1.15	1.80	2.38
2 \times Wild type	24	0.95	1.60	2.25
1/2 \times Wild type	30	0.92	1.40	2.10
Wild type	30	0.78	1.40	2.00
2 \times Wild type	30	0.60	1.25	1.85

peratures $>34^\circ\text{C}$. Both mutants show some α -factor-dependent receptor clearance at permissive temperature with *end4* cells clearing somewhat more receptor activity than *end3* cells, but do not show any clearance at 35°C. The accumulation of LY by *end3* and *end4* mutants mirrors their α -factor-dependent receptor clearance characteristics. *end3* strains are very defective for vacuolar LY accumulation at any temperature (Fig. 3). *End4* strains accumulate LY in the vacuole in more cells at 24 than at 37°C. There are apparent quantitative differences between the results of the α -factor internalization assays and the α -factor receptor clearance assays for the *end4* mutant. This could be explained in the following manner: In the α -factor internalization assay only a small percentage of receptors were occupied ($<10\%$ as calculated from the α -factor concentration and the receptor K_d) and therefore triggered to undergo endocytosis. If the pathway was only slightly active (e.g., 10% of wild-type efficiency), but the small number of receptors could be efficiently targeted into the pathway only a relatively small defect would have been seen. In the receptor clearance assay, we examined the location of all of the α -factor receptors which had been saturated with pheromone. In this case, the large number of receptors to be internalized were not able to be completely loaded into the defective pathway. This interpretation would also be applicable to the LY accumulation experiments. The *end4* mutant was apparently more defective for LY accumulation than for α -factor uptake. The LY accumulation assay measures net nonspecific endocytic uptake and if the pathway functions at a low efficiency only a small amount of accumulation would occur. In any event it is clear that both mutants are defective for endocytosis and that the *end4* mutant shows some endocytic capacity at 24°C. These are the first two endocytic mutants to show both α -factor internalization, receptor clearance and LY accumulation defects and therefore indicate that common proteins service the entry of fluid-phase and receptor borne markers in yeast.

Economy of function also appears to operate among participants of the secretory and endocytic pathways in yeast. One protein, Sec18p, the yeast homologue of the *N*-ethylmaleimide-sensitive fusion protein (NSF) (*N*-ethylmaleimide-sensitive fusion protein), functions directly in both pathways. NSF is necessary for intra-Golgi transport, ER to Golgi transport, and endosome fusion as shown from in vitro assays using mammalian cell extracts (Wilson et al., 1989;

Beckers et al., 1989; Diaz et al., 1989). In vivo, the *SEC18* gene has been shown to be essential for ER-Golgi transport, transport events within the Golgi, and for delivery of endocytic content and α -factor to the vacuole (Novick et al., 1980; Graham and Emr, 1991; Riezman, 1985; Riezman et al., 1992). In addition, evidence has been provided suggesting that endocytosis may be obligatorily coupled with the latest steps of secretion. It was postulated that the latter steps of the secretory pathway could be used to replenish components needed at the plasma membrane for endocytosis (Riezman, 1985). Our results show that the secretory pathway is not obligatorily coupled to the endocytic pathway. Invertase secretion and maturation of CPY are not blocked in the *end* mutants at restrictive temperatures. Furthermore, the endocytic defect in the *end3* mutant is manifested at the restrictive temperature as well as the permissive temperature. Since cells cannot grow without the secretory pathway the *end3* mutant cannot be blocked in secretion. This, however, does not mean that proteins used in the secretory pathway (in fusion of secretory vesicles with the plasma membrane) are not recycled for reuse via endocytosis. Even if their recycling was blocked, another port of entry into this pathway exists from newly synthesized material. This is not the case for a potential transmembrane protein required at the cell surface for endocytosis. Its only port of entry to the plasma membrane is via the secretory pathway and if the latter steps of this pathway are blocked this could lead to a slow down or stop of endocytosis. Our findings suggest that endocytosis may be obligatory for cell growth at 37°C, but not 24°C in yeast and show that under certain conditions yeast cells can grow and divide with considerably reduced endocytosis. There are different possible explanations for these observations. Perhaps endocytic uptake is necessary for the removal of heat denatured proteins from the plasma membrane for subsequent degradation in the vacuole. Another explanation could be that without endocytosis a membrane imbalance is generated when secretion rates get too high.

The endocytic defect in *end4* cells shows some temperature sensitivity. All three measures of endocytosis, α -factor uptake, receptor clearance, and LY accumulation, although to varying degrees suggest that the End4 mutant protein is a temperature-sensitive protein that is partially functional at 24°C, but is rendered irreversibly inactive at 34°C. Mapping and elucidation of the specific mutation in *end4* could provide interesting information concerning the functional domains of this protein.

Identification of conditions stalling α -factor while in transit from plasma membrane to vacuole by Singer et al. (1990) allowed the *end4* mutant block to be placed temporally within the endocytic pathway. By binding α -factor under conditions where no internalization occurs and subsequently shifting cells to 15°C for 20 min to allow uptake, internalized but undegraded α -factor can be recovered in membrane-bounded intermediate compartments (Singer and Riezman, 1990). Upon shift from 15°C to higher temperatures in wild-type cells, this undegraded α -factor can be chased into degraded α -factor indicating delivery of the ligand to the vacuole. The same results are found when *end4* cells, allowed to internalize α -factor at 15°C for 20 min, are shifted to restrictive temperature and assayed for degradation (Fig. 7). Transit of α -factor from the 15°C compartment to the vacuole is not affected by the *end4* mutation since degradation occurs normally when cells are shifted from 15°C to the

restrictive temperature. The total amount of α -factor internalized as indicated quantitatively by the pH 1/pH 6 ratios in parentheses below the lanes and qualitatively by the consistent amount of α -factor seen in the pH 6 lanes of Fig. 7, changes very little once *end4* cells are shifted from 15 to 34°C. This provides a nice internal control for the presence of the endocytic defect throughout this experiment and dispels the possible criticism that without an immediate block in internalization α -factor could continue to leak through the pathway resulting in a false positive. *END4*, therefore, must act exclusively at the earliest step(s) of the internalization pathway, including or preceding the formation of the 15°C intermediate, and not the later steps involving the transit of α -factor from the 15°C compartment to the vacuole. In addition, its role in the endocytic pathway must be rather direct because no preincubation at nonpermissive temperature is needed to install the phenotype.

We believe the data presented in this paper conclusively demonstrate that *end3* and *end4* are the first yeast endocytic mutants defective in the earliest step(s) of the internalization process. Another yeast mutant that shows a partial defect in α -factor uptake is the clathrin heavy chain mutant (*chcl*; Payne et al., 1988). A *chcl* mutant completely lacking clathrin heavy chain was still capable of internalizing α -factor with ~35–50% efficiency. Therefore, the clathrin heavy chain seems to be less essential for endocytic uptake in yeast than the proteins defined by the *END3* and *END4* genes. The cloning of these genes and further characterization of these mutants should lead to identification of proteins that are essential for the endocytic pathway and hopefully expand our understanding of the mechanism of the early steps of endocytosis. The demonstration that α -factor trapped along the endocytic pathway at nonpermissive temperature in the *end4* mutant can be chased into the cell at permissive temperature suggests that the α -factor was not diverted off the normal endocytic route. This block in endocytic transport could help identify new intermediates in the process and might aid in identifying proteins that interact with the trapped α -factor receptor.

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References

- Anderson, R. G. W., M. S. Brown, and J. L. Goldstein. 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell*. 10:351–364.
- Bartles, J. R., H. M. Feracci, B. Stieger, and A. L. Hubbard. 1987. Biogenesis of the rat hepatocyte plasma membrane in vivo: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. *J. Cell Biol.* 105:1241–1251.
- Beckers, C. J. M., M. R. Block, B. S. Glick, J. E. Rothman, and W. E. Balch. 1989. Vesicular transport between endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. *Nature (Lond.)*. 339:397–398.
- Beguinet, L., M. Lyall, M. C. Willingham, and I. Pastan. 1984. Down-regulation of the epidermal growth factor receptor in KB cells is due to receptor internalization and subsequent degradation in lysosomes. *Proc. Natl.*

- Acad. Sci. USA.* 81:2384-2388.
- Blumer, K., and J. Thorner. 1990. β and γ subunits of a yeast guanine nucleotide-binding protein are not essential for membrane association of the α subunit but are required for receptor coupling. *Proc. Natl. Acad. Sci. USA.* 87:4363-4367.
- Chavrier, P., R. G. Parton, H.-P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell.* 62:317-329.
- Chen, M. S., R. A. Obar, C. C. Schroeder, T. W. Austin, C. A. Poodry, S. C. Wadsworth, and R. B. Vallee. 1991. Multiple forms of dynamin are encoded by *shibire*, a *Drosophila* gene involved in endocytosis. *Nature (Lond.)* 351:583-586.
- Chvatchko, Y., I. Howald, and H. Riezman. 1986. Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell.* 46:355-364.
- Colbaugh, P. A., C.-Y. Kau, S.-P. Shia, M. Stookey, and R. K. Draper. 1988. Three new complementation groups of temperature-sensitive Chinese hamster ovary cell mutants defective in the endocytic pathway. *Somatic Cell Mol. Genet.* 14:499-507.
- Diaz, R., L. S. Mayorga, P. J. Weidman, J. E. Rothman, and P. D. Stahl. 1989. Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport. *Nature (Lond.)* 339:398-400.
- Dulic, V., and H. Riezman. 1989. Characterization of the END1 gene required for vacuole biogenesis and gluconeogenic growth of budding yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1349-1359.
- Dulic, V., M. Egerton, I. Elguindi, S. Raths, B. Singer, and H. Riezman. 1990. Yeast endocytosis assays. *Methods Enzymol.* 194:697-710.
- Gasser, S. M., G. Daum, and G. Schatz. 1982. Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. *J. Biol. Chem.* 257:13034-13041.
- Goldstein, A., and J. O. Lampen. 1975. β -D-fructofuranoside fructohydrolase from yeast. *Methods Enzymol.* 42:504-511.
- Graham, T. R., and S. D. Emr. 1991. Compartment organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (NSF) mutant. *J. Cell Biol.* 114:207-218.
- Harding, C. V., and E. R. Unanue. 1989. Antigen processing and intracellular Ia. Possible roles of endocytosis and protein synthesis in Ia function. *J. Immunol.* 142:12-19.
- Hemmaplardh, D., and E. H. Morgan. 1976. Transferrin uptake and release by reticulocytes treated with proteolytic enzymes and neuraminidase. *Biochim. Biophys. Acta.* 426:385-398.
- Jenness, D. D., A. C. Burkholder, and L. H. Hartwell. 1983. Binding of α -factor pheromone to yeast a-cells: chemical and genetic evidence for an α -factor receptor. *Cell.* 35:521-529.
- Jenness, D. D., and P. Spatrick. 1986. Down regulation of the α -factor pheromone receptor in *S. cerevisiae*. *Cell.* 46:345-353.
- Karin, M., and B. Mintz. 1981. Receptor-mediated endocytosis of transferrin in developmentally totipotent mouse teratocarcinoma stem cells. *J. Biol. Chem.* 256:3245-3252.
- Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483-525.
- Kosaka, T., and K. Ikeda. 1983. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of *Drosophila melanogaster shibire^{ts}*. *J. Cell Biol.* 97:499-507.
- Krieger, M., D. Kingsley, R. Sege, L. Hobbie, and K. Kozarsky. 1985. Genetic analysis of receptor-mediated endocytosis. *Trends Biol. Sci.* 10:447-452.
- Kühn, L. C., and J.-P. Kraehenbuhl. 1982. The sacrificial receptor-translocation of polymeric IgA across epithelia. *Trends Biochem. Sci.* 7:299-302.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Matter, K., M. Brauchbar, K. Bucher, and H.-P. Hauri. 1990. Sorting of endogenous membrane proteins occurs from two sites in cultured human intestinal epithelial cells (Caco-2). *Cell.* 60:429-437.
- Merion, M., P. Schlesinger, R. M. Brooks, J. M. Moehring, T. J. Moehring, and W. S. Sly. 1983. Defective acidification of endosomes in Chinese hamster ovary cell mutants "cross-resistant" to toxins and viruses. *Proc. Natl. Acad. Sci. USA.* 80:5315-5319.
- Novick, P. C., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for posttranslational events in the yeast secretory pathway. *Cell.* 21:205-215.
- Obar, R. A., C. C. Collins, J. A. Hammarback, H. S. Shpetner, and R. B. Vallee. 1990. Molecular cloning of the microtubule-associated mechanochemical enzyme dynamin reveals homology with a new family of GTP-binding proteins. *Nature (Lond.)* 347:256-261.
- Payne, G. S., D. Baker, E. van Tuinen, and R. Schekman. 1988. Protein transport to the vacuole and receptor-mediated endocytosis by clathrin heavy chain-deficient yeast. *J. Cell Biol.* 106:1453-1461.
- Pearse, B. M. F., and M. S. Robinson. 1990. Clathrin, adaptors and sorting. *Annu. Rev. Cell Biol.* 6:151-171.
- Reneke, J. E., K. J. Blumer, W. E. Courchesne, and J. Thorner. 1988. The carboxy-terminal segment of the yeast α -factor receptor is a regulatory domain. *Cell.* 55:221-234.
- Riezman, H. 1985. Endocytosis in yeast: several of the yeast secretory mutants are defective in endocytosis. *Cell.* 40:1001-1009.
- Riezman, H., S. Raths, M. Rissi, B. Singer, and B. Zanolari. 1992. Receptor-mediated endocytosis of α -factor by *S. cerevisiae* a cells. In *Endocytosis: From Cell Biology to Health, Disease and Therapy*. P. J. Courtroy, editor. NATO ASI Series, Vol. H 62. Springer-Verlag, Berlin, Germany. 467-473.
- Robbins, A. R., S. S. Peng, and J. L. Marshall. 1983. Mutant Chinese hamster ovary cells pleiotropically defective in receptor-mediated endocytosis. *J. Cell Biol.* 96:1064-1071.
- Robinson, J. S., D. J. Klionsky, L. M. Banta, and S. D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8:4939-4948.
- Rothman, J. H., and T. H. Stevens. 1986. Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. *Cell.* 47:1041-1051.
- Rothman, J. H., I. Howald, and T. H. Stevens. 1989. Characterization of genes required for protein sorting and vacuolar function in the yeast *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2057-2065.
- Sherman, F., G. Fink, and C. Lawrence. 1974. *Methods in yeast genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Singer, B., and H. Riezman. 1990. Detection of an intermediate compartment involved in transport of α -factor from plasma membrane to the vacuole in yeast. *J. Cell Biol.* 110:1911-1922.
- Stevens, T. H., B. Esmon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* 30:439-448.
- Stevens, T. H., J. H. Rothman, G. S. Payne, and R. Schekman. 1986. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. *J. Cell Biol.* 102:1551-1557.
- Wilson, D., C. Wilcox, G. Flynn, E. Chen, W.-J. Kuang, W. Henzel, M. Block, A. Ullrich, and J. E. Rothman. A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature (Lond.)* 339:355-359.