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 MATα and MATα-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 cosegregate 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 35S-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; Beguinet 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), 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

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, endl and end2 (Chvatchko et al., 1986). Further characterization of the endl 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 35S-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 (MATa his4 leu2 ura3 bar1 Δ trpl::URA3 bar-1), RH144-3B (MATa his4 leu2 ura3 bar1 Δ endl::LEU2), RH266-3D (MATa end3 ura3 leu2 his4 ldr1-1), RH266-IC (MATa end3 ura3 leu2 his4 ldr1-1), and RH765 (MATa ura3 his4 leu2 trpl::URA3 bar-1). RH449 (Mata his4 leu2 ura3 lys2 ldr2-1) transformed with pDA6300 (a 2μ-based plasmid containing the MFA1, STE13, and LEU2 genes, provided by D. Barnes and J. Thorner, University of California, Berkeley, CA) was used for 35S-α-factor production as described previously (Dulic and Riezman, 1989; Dulic et al., 1990). All yeast strains were grown in complete medium (YPAD) containing 1% yeast extract, 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 in which 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 YPAD with the addition of 10 μM sodium azide, 10 mM potassium fluoride and 10 mM p-tyrosyl-l-argininemethyl ester (TAME; Janesh et al., 1983; Jenness and Spatrick, 1986). Carrier-free H235SO4 was obtained from New England Nuclear (Boston, MA). Biosynthetically labeled 35S-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 Carboxyazole 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 × 10^6 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 twice with 5% sodium thiosulfate and finally resuspended in YPUD (1 × 10^6 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 YPUD for single colony isolation. After 2 d of growth at 24°C the colonies were replica plated onto two YPUD 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 × 10^9 cells/ml at 24°C in YPUD. Once harvested the cells were resuspended to a density of 1 × 10^9 cells/ml in YPUD and preincubated 10 min at 24° or 37°C in a shaking water bath. 35S-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) to the scintillation counting vials. Scintillation counting was done with a Packard Tricarb liquid scintillation counter. Mutants that failed to incorporate 35S into TCA-precipitable counts were discarded. Two mutants, RH1565 (MATa end3 ura3 his4 ldr2-1) and RH1754 (MATa end4 ura3 his4 ldr1-1) were found to be the first 113 temperature-sensitive clones screened. Before experimentation with these mutants was continued, they were backcrossed twice to RH765, a strain that is isogenic to the parent, RH144-3D and the tetrad were dissected and analyzed using standard genetic techniques (Sherman et al., 1983). Two segregants from the second backcross, RH2656-1D(end3) and RH2658-1C(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 35S-α-factor.

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Downregulation of receptor sites was assayed in end3, end4, and wild-type strains essentially as described (Jenness and Spradnick, 1986). In brief, strains were grown overnight in YPUAD medium at 24°C to a density of 1 x 10^6 cells/ml. After harvesting, two 120-ml cultures containing 5 x 10^7 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 x 10^8 cells) were withdrawn and collected on nitrocellulose filters. Filters containing cells were washed twice 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 μl 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 replicate binding reactions were set up for Stage II of the experiment as follows. In this procedure, 35S-α-factor (30,000 cpm) was added to 100 μl of cells (2 x 10^7 cells) in inhibitor medium for the test reactions and 35S-labeled α-factor (30,000 cpmp), 2 x 10^7 cells and unlabeled α-factor to a final concentration of 2 x 10^-3 M were combined for the control reactions.

After an incubation of 30 min at 25°C, 90 μl (representing 1.5 x 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 scintillator. Those counts which could be competed by 2 x 10^-3 M unlabeled α-factor were considered to represent specific binding.

LY-CH 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 in 10^8 cells/ml for the assay. One aliquot of cells was 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) and a Zeiss Axiophot microscope. Exposure times 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 x 10^6 cells/ml. The culture was shifted to 37°C slowly by simply readjusting the temperature setting on the water bath from 24 to 37°C. 10 min after the water bath reached 37°C, 3.5 x 10^7 cells were removed and put on ice as an uninduced control. The remaining cells were harvested by centrifugation, washed twice with water equilibrated to 37°C and resuspended to the original volume (8 x 10^6 cells/ml) in prewarmed (37°C) YPUAD containing only 0.1% glucose. At the indicated times, 3.5 x 10^7 cells were removed, pelleted at 4°C, and resuspended in 500 μl ice-cold 10 mM sodium azide. From each 500 μl aliquot, 250 μl were removed for the determination of the amount of external invertase present and the other 250 μl 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 μl 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), end4 (RH44-3B), or wild type (RH44-3D) 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 x 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 medium and finally resuspended to 2 x 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 μl of H35SO4 (40 mM/mil). 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 μg/ml to initiate the period of chase. At 0, 5, 10, 15, and 30 min of chase, 500 μl of the culture was removed to microtubes on ice containing 50 μl of 0.2 M NaN3/0.2 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 immunoprecipitates were applied to poly L-lysine-coated microscopic slides. Cells were converted to spheroplasts using lyticase (Stevens et al., 1986) for 45 min at 30°C in the presence of 20 mM each NaN3 and NaF. The spheroplast extracts were collected (3,000 g, 5 min) and the supernatant (periplasm) was 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 immunoprecipitates 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 mM 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 Dulic and Riezman (1989). end4 (RH268-1C) and wild-type cells (RH44-3D) grown at 24°C to <10^7 cells/ml were harvested and allowed to bind 35S-α-factor (20,000 cpm/10^6 cells) on ice for 1 h. 100-μl duplicate aliquots were removed to (a) determine the total amount of cell-associated radioactivity and the internalized amount of 35S-α-factor by the internalization assay and (b) evaluate the condition of the 35S-α-factor, if internalized, as 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 x 10^6 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 negative to determine the internalization sites of 35S-α-factor were pelleted again for 3 min at 3,500 rpm, 4°C and resuspended to the same density (1 x 10^6 cells/ml) in medium prewarmed at 34°C. At the indicated times after shift to 34°C, 100-μl 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 x 20 cm, Merck, Germany) and the butanol:propionic acid:water, 50:25:35 (vol/vol/vol) solvent system used previously (Dulic et al., 1990). ENHANCE 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

RH44-3D, RH266-1D, or RH268-1C cells were grown to early logarithmic phase and counted. The cells (5 x 10^6/ml = 1 x 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 x 16 mm; Greiner Labortechnik, Nütingen, 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 μl) of α-factor to one side of 6.3-mm filter disks (Schleicher and Schuell, F clerk, Switzerland) which were then placed, a-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 a-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 35S-a-factor bound at 37°C could be internalized after a 30 min incubation at that temperature. Control incubations of cells with 35S-a-factor at 24°C revealed that one mutant, end3, was also unable to internalize a-factor at 24°C, permissive growth temperature. The other mutant, end4, internalized ~70% of the total bound a-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 a-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 (RHI44-3D) 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 a-factor Internalization, Receptor Downregulation, and LY Accumulation

To determine whether these mutants contain true blocks in the endocytosis of a-factor or only affect the rate at which a-factor is taken up, the internalization of 35S-a-factor was followed over time at both permissive and restrictive growth temperatures in each mutant. For these studies, 35S-a-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 35S-a-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 a-factor, respectively. The results of these experiments are shown in Fig. 1. a-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 35S-a-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 35S-a-factor at 24°C, internalization was again virtually complete by 30 min (>90% of maximum), however the amount of bound a-factor internalized by end4 was less than wild type (72% that of wild type). At restrictive temperature end4 cells, like end3 cells, internalized very little bound a-factor. a-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 a-factor uptake block in end4 cells requires no preincubation at nonpermissive temperature (234°C; data not shown). Using initial rates of uptake calculated from experiments performed on cells with 35S-a-factor prebound at 0°C (pulse-chase protocol; Dulic et al., 1990), the half-times of maximal internalization (t1/2) of a-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 a-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 a-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 a-factor and one half received no a-factor at all. At various times, aliquots were withdrawn, the cells washed extensively with YPUAD medium containing the metabolic inhibitors NaF and Na3 dilitated and held at 25°C to allow dissociation of surface-bound a-factor. The binding capacity of each cell sample was subsequently determined by incubation with 35S-a-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 a-factor–independent loss of cell surface binding activity than at 37°C. As shown in Fig. 2, upon addition of a-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
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). Endocytosis 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 μm.

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 colorometric 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, pl 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 H235SO4 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
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 pl, p2, and mCPY were the same in the three strains. All of the pl 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 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 (pl, 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).

The α-factor Internalization Defect in end4 Is Reversible

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 35S-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 35S-α-factor that was bound. end4 cells first preincubated at 34°C, then incubated with 35S-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 (Δ), 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 allow 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 MA1a 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 temperatures >34°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 Kd) 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 temporarily 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.

We wish to thank Paul Jeno for expert advice concerning α-factor purification, Mark Egerton for enlightening discussions and lab humor, Linda Hicke and Alan Munn for critical reading of the manuscript and the members of the Department of Biochemistry at the Biocenter for critical evaluation of this work while in progress.

This work was supported by a grant from the Swiss National Science Foundation and by the Kanton Basel-Stadt.

Received for publication 20 July 1992 and in revised form 21 September 1992.

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