

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 cosegregates 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; 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), 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 on 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, 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 ethyl-methanesulfonate (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 (MATb his4 leu2 ura3 barl-l), RH144-3B (MATb his4 leu2 ura3 barl-l endl-LEU2), RH266-3D (MATb end3 ura3 leu2 his4 barl-l), RH266-IC (MATb end3 ura3 leu2 his4 barl-l), and RH765 (MATb ura3 his4 leu2 trpl::URA3 barl-l). RH449 (Mats his4 leu2 ura3 lys2 barl-l) transformed with pDA6300 (a 2μ-based plasmid containing the MFA1, STE1, 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 (YPUD) containing 1% yeast extract, 2% peptone, 40 μg/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 YPUD with the addition of 10 mM sodium azide, 10 mM potassium fluoride and 10 mM p-rosyl-l-argininemethyl ester (TAME; Jenness 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 Carboxydrazide 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 YPUD to stationary phase. After harvesting by centrifugation, the cells were prepared for mutagenesis by resuspension to 1.4 × 107 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 × 107 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 × 107 cells/ml at 24°C in YPUD. Once harvested the cells were resuspended to a density of 1 × 107 cells/ml in YPUD and preincubated 20 min at 24°C or 37°C in a shaking water bath. 35S-α-factor (1 × 107 cpm/107 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 each scintillation counting vial. After scintillation counting, samples were counted a second time at different light 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 × 107 cells were harvested and resuspended in 40 μl of no-sulfate minimal medium and allowed to preincubate 10 min at either 24°C or 37°C. 25 μl of SD no-sulfate medium containing 3 μCi H235SO4 was then added and the incorporation of 35SO42- 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 35S into TCA-precipitable counts were discarded. Two mutants, RH1565 (MATb end3 ura3 his4 barl-l) and RH1574 (MATb end4 ura3 his4 barl-l) were found in 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 was dissected and analyzed using standard genetic techniques (Sherman et al., 1983). Two segregants from the second backcross, RH266-3D(end3) and RH268-IC(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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Regensdorf, Switzerland) was used to coat the plates for fluorography using Kodak XAR-5 film (Eastman Kodak Co.).

**CPY Biogenesis**

Radiolabeling of cells, either end3 (RH266-1D), end4 (RH268-1C), end1 (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). Yeast cultures grown overnight at 24°C were harvested and resuspended in 50 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 duplicate binding reactions were set up for Stage II of the experiment as follows. In this procedure, 35S-α-factor (50,000 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 cpm), 2 x 10^7 cells and unlabeled α-factor to a final concentration of 2 x 10^-5 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 Emlusilfer-Safe scintillant. Those counts which could be competed by 2 x 10^-5 M unlabeled α-factor were considered to represent specific binding.

**α-factor Degradation Assay**

α-factor degradation assays were performed as described by Dulic and Riezman (1989). Cells (2 x 10^7 cells) in SD low-sulfate medium containing 2 mg/ml BSA. Cells (2.5 ml) were preincubated 10 min at 37°C before adding 25 μl of H_35SO_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 μg/ml to initiate the period of chase. At 0, 5, 10, and 30 min of chase, 300 μl of the culture was removed for the determination of total (internal + external) CPY radioactivity. Those counts which could be competed by 2 x 10^-5 M unlabeled α-factor were considered to represent specific binding.

**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 YPUD medium and then mixed with YPUD medium in 0.8% 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 waterbath from 24 to 37°C. Once 35°C was reached, LY was added to 4 mg/ml. To the other aliquot LY was 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 1% gels with fluorographic enhancement using 1 M sodium salicylate. The films were quantified by scanning using a densitometer (Molecular Dynamics, Sunnyvale, CA).

**Invertase Secretion Assay**

Yeast cells were grown overnight at 24°C in YPUD medium containing antisence plus approximately 8 x 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. At 20 rain, the cells were pelleted again for 3 rain at 3,500 rpm, washed with YPUD and then mixed with YPUD medium in 0.8% 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 waterbath from 24 to 37°C. Once 35°C was reached, LY was added to 4 mg/ml. To the other aliquot LY was 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 1% gels with fluorographic enhancement using 1 M sodium salicylate. The films were quantified by scanning using a densitometer (Molecular Dynamics, Sunnyvale, CA).

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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 35S-α-factor bound at 37°C could be internalized after a 30 min incubation at that temperature. Control incubations of cells with 35S-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 MAHΔ 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 deficits of the two mutants are also recessive.

end3 and end4 Mutants Are Defective in α-factor Internalization, Receptor Downregulation, and αY 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 35S-α-factor was followed over time at both permissive and restrictive growth temperatures in each mutant. For these studies, 35S-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 35S-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 35S-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 35S-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 temperature 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 non-permissive temperature (>34°C; data not shown). Using initial rates of uptake calculated from experiments performed on cells with 35S-α-factor prebound at 0°C (pulse-chase protocol; Dulic et al., 1990), the half-times of maximal internalization (t1/2) 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 MAHΔ 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 NaF, and Na3, 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 35S-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...
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 labeling. 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 did.
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.

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
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.

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 (A), 34°C medium (●), or 24°C medium containing cycloheximide (Δ) (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.

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.
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 33S-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 33S-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 MAIΔ 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 endocytotic 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 a-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 a-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 last 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, a-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 a-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 a-factor under conditions where no internalization occurs and subsequently shifting cells to 15°C for 20 min to allow uptake, internalized but degraded a-factor can be recovered in membrane-bound intermediate compartments (Singer and Riezman, 1990). Upon shift from 15°C to higher temperatures in wild-type cells, this unprocessed a-factor can be chased into degraded a-factor indicating delivery of the ligand to the vacuole. The same results are found when end4 cells, allowed to internalize a-factor at 15°C for 20 min, are shifted to restrictive temperature and assayed for degradation (Fig. 7). Transit of a-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 a-factor internalized as indicated quantitatively by the pH 1/pH 6 ratios in parentheses below the lanes and qualitatively by the consistent amount of a-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 a-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 a-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 a-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 a-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 a-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 a-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 a-factor receptor.

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