Saccharomyces cerevisiae mutants lacking a functional vacuole are defective for aspects of the pheromone response

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Summary

The *end1* mutant belongs to a group of four vacuolar protein sorting mutants (class C *vps*) that lack a morphologically distinguishable and functional vacuole. These mutants share several other phenotypes, such as the inability to grow at 37°C or on nonfermentable carbon sources. We show that, as in the case of the *end1* mutant, *vps16*, *vps18* and *vps33* mutants all internalize but do not degrade α-factor. In addition, all four mutants are defective for α-factor-induced projection formation to the same extent. A more detailed investigation of pheromone response in the *end1* mutant reveals that one aspect of the early response (induction of *FUS1*) is as defective as late responses (cell cycle arrest and projection formation). In contrast, another measure of the early response (induction of *STE2*) is normal. These data suggest that the biogenesis of a functional vacuole is necessary for optimal response to pheromone.

Key words: *end1*, α-factor, gene induction, yeast vacuole biogenesis, *vps* mutants.

Introduction

Mating between haploid partners of *Saccharomyces cerevisiae* is initiated by secreted peptide pheromones, **a**-factor and α-factor. These pheromones bind receptors on the cell surface of the mating partner and stimulate transcription of genes involved in conjugation, induce cell surface agglutinins, coordinate the cells for cell and nuclear fusion, arrest the cell cycle at 'START', and produce a characteristic, pear-shaped cell called a 'shmoo' (reviewed by Cross *et al.* 1988; and Herskowitz, 1989). Signal transduction requires the STE2 and STE3 proteins, which are the receptors for α- and **a**-factor, respectively (Jenness *et al.* 1983; Hagen *et al.* 1986; Nakayama *et al.* 1985). STE2 and STE3 have a predicted transmembrane topology that is similar to other receptors that transduce extracellular signals by interacting with G proteins (Dietzel and Kurjan, 1987; Dixon *et al.* 1986; Miyajima *et al.* 1987; Whiteway *et al.* 1989).

After binding to the receptor, α-factor is internalized by **a** cells in a time-, temperature- and energy-dependent manner (Chvatchko *et al.* 1986; Jenness and Spatrick, 1986). α-Factor is subsequently degraded (Chvatchko *et al.* 1986), most likely in the vacuole (Dulić and Riezman, 1989; Singer and Riezman, 1990), by a mechanism that requires PEP4, the vacuolar hydrolase (Jones, 1977; Jones *et al.* 1982; Ammerer *et al.* 1986; Woolford *et al.* 1986). The yeast vacuole is similar in many respects to lysosomes from mammalian cells. Both are acidic compartments that house a variety of hydrolytic enzymes and are involved in degradative and nutrient recycling functions. In addition, the yeast vacuole stores basic amino acids, phosphate and inorganic ions that are mobilized upon starvation (Matile, 1978; Wiemken *et al.* 1979).

The *Saccharomyces cerevisiae end1* mutant was isolated because it fails to accumulate a fluorescent endocytic marker Lucifer Yellow CH in the vacuole (Chvatchko *et al.* 1986). In fact, the *end1* mutant lacks a functional vacuole, on the basis of microscopic and biochemical findings (Chvatchko *et al.* 1986; Dulić and Riezman, 1989; Westenberg *et al.* 1989; Robinson *et al.* 1988). Four mutants identified by Emr and coworkers, which are defective in vacuolar protein targeting (*vpt11, 16, 18* and *33*), also lack a vacuole (Banta *et al.* 1988; Robinson *et al.* 1988). One, *vpt11*, is allelic to *end1*, *pep5* (Jones, 1977) and *vpl9* (Rothman *et al.* 1989). Although a common nomenclature has been suggested for mutants defective in vacuole biogenesis, *vps* (vacuole protein sorting; Rothman *et al.* 1989), we continue to refer to *end1* by its previous name to avoid confusion and to distinguish between *end1* and *vps11* alleles. The four *vps* mutants (termed class C) share several common features: all are osmotically sensitive, secrete their vacuolar proteases, do not grow at 37°C, and grow poorly on non-fermentable carbon (C) sources (Banta *et al.* 1988; Robinson *et al.* 1988; Dulić and Riezman, 1989). This common phenotype suggests a role for the vacuole in cellular homeostasis.

The *end1* mutant also shows a defect both in response to pheromone and in mating (Chvatchko *et al.* 1986). Initial studies suggested that α-factor uptake was defective in the mutant (Chvatchko *et al.* 1986). However, more recent data clearly demonstrated that *end1* mutant cells internalize α-factor normally but the internalized pheromone is not degraded (Dulić and Riezman, 1989). The α-factor

Table 1. *Yeast strains used in this study*

Strain	Genotype	Source
X2180-1A	*MAT***a**, *SUC2, mal, gal2, CUP1*	Yeast Genetic Stock Center, Berkeley
RH36-4	*MAT***a**, *lys2, end1*	Laboratory collection
RH444	*MAT***a**, *his4, leu2, ura3, bar1-1* / *MATα, his4, leu2, ura3, bar1-1*	Laboratory collection
RH144-3A	*MATα, his4, leu2, ura3, bar1-1*	Laboratory collection
RH144-3B	*MATα, his4, leu2, ura3, bar1-1, end1Δ1::LEU2*	Laboratory collection
RH144-3C	*MATα, his4, leu2, ura3, bar1-1, end1Δ1::LEU2*	Laboratory collection
RH144-3D	*MAT***a**, *his4, leu2, ura3, bar1-1*	Laboratory collection
RH144-3A-trp	RH144-3A, *trp1::URA3*	This work
RH144-3B-trp	RH144-3B, *trp1::URA3*	This work
RH144-3C-trp	RH144-3C, *trp1::URA3*	This work
RH144-3D-trp	RH144-3D, *trp1::URA3*	This work
RH732	RH144-3D, *pep4::URA3*	Laboratory collection
RH151-7B	*MAT***a**, *his4, leu2, ura3*	Laboratory collection
RH151-7Be	RH151-7B, *end1Δ1::LEU2*	This work
SEY6211	*MAT***a**, *ura3-52, leu2-3, -112, his3-Δ200, trp1-Δ901, ade2-101, suc2Δ9*	S. Emr, Pasadena
SEY6211-bar1	SEY6211, *bar1::LYS2*	This work
SEY1474	SEY6211, *vps11*	S. Emr, Pasadena
SEY1490	SEY6211, *vps16*	S. Emr, Pasadena
SEY1490-bar1	SEY1490, *bar1::LYS2*	This work
SEY1477	SEY6211, *vps18*	S. Emr, Pasadena
SEY1477-bar1	SEY1477, *bar1::LYS2*	This work
SEY15194	SEY6211, *vps33*	S. Emr, Pasadena
SEY15194-bar1	SEY15194, *bar1::LYS2*	This work
SEY6211*e*	SEY6211, *end1Δ1::LEU2*	This work
SEY6211-rho^0	SEY6211, rho^0	This work

degradation defect of the *end1* mutant could be attributed either to a defect in vesicular traffic in the endocytic pathway or to the lack of a functional vacuole.

Here we show that all of the class C *vps* mutants internalize but do not degrade α-factor. Furthermore, we show that all four *vps* mutants are defective for aspects of late pheromone response whereas the *pep4* mutant is not. Some aspects of the early pheromone response of the *end1* mutant have been studied in detail. While pheromone-induced transcription of *STE2* in *end1* is unaltered, *end1* cells require a tenfold higher pheromone concentration to induce transcription of *FUS1*, whose product is necessary for cell fusion during mating (Trueheart *et al.* 1987). Our data imply that optimal pheromone response requires vacuole biogenesis or function.

Materials and methods

Yeast strains, media and transformation

The *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. *vps* mutants (Banta *et al.* 1988; Robinson *et al.* 1988) were kindly provided by Dr S. Emr (Cal. Tech., Pasadena, CA). A *pep4* null mutant was obtained by disrupting the *PEP4* gene in RH144–3D by a one-step gene disruption (Rothstein, 1983) using the *Eco*RI fragment of plasmid pTS15 (kindly provided by T. Stevens, University of Oregon, Eugene, OR). All SEY6211-derived strains were plated onto α-aminoadipic acid-containing plates (Chattoo *et al.* 1979) and spontaneous *lys2* derivatives were selected. These strains were then rendered *bar1* by one-step gene disruption using the *Eco*RI fragment of pEK-3. This plasmid contains the *BAR1* gene disrupted with the *LYS2* gene (E. Kubler, Biozentrum, Basel). The *BAR1* gene encodes a secreted protease that degrades α-factor extracellularly (MacKay *et al.* 1988). The other strains are described previously (Dulić and Riezman, 1989). Yeast cells were cultured in YPUAD medium (1% yeast extract, 2% peptone, 2% glucose, 30 mg ml^{-1} each of uracil and adenine) or SD medium using $(NH_4)_2SO_4$ as N source, 2% glucose as C source, and supplemented with the necessary bases or amino acids (Dulić *et al.* 1990). Solid media were prepared with 2% agar. Yeast transformation was performed as described (Ito *et al.* 1983).

Nucleic acid techniques and plasmids

DNA manipulations, transformation and growth of *Escherichia coli* SE10 strain (Emr *et al.* 1986) were performed as described (Maniatis *et al.* 1982). For Northern (RNA) blot analysis the following DNA fragments served as probes: for *END1*, 1.5-kb *Eco*RI–*Xba*I fragment from pVD01 (Dulić and Riezman, 1989); for *STE2* and *URA3*, pIU-STE2 (1.1 kb and 1.7 kb *Hin*dIII fragments containing the *URA3* and *STE2* genes, respectively, in the *Hin*dIII and *Pvu*II sites of pBR322; constructed by M. Moya) was linearized with *Bam*HI; for *FUS1*, *BIK1* and *URA3*, 1.4-kb *Eco*RI and 1.7-kb *Eco*RI–*Hin*dIII fragments from the plasmid pSB234, kindly provided by J. Trueheart (Trueheart *et al.* 1987). Plasmid YCpSTE2-B2 containing the *STE2* gene, *TRP1* and *CEN3*, was kindly provided by N. Nakayama (University of Tokyo, Tokyo). Isolation of poly(A)$^+$ RNA was performed as described (Jensen *et al.* 1983). Poly(A)$^+$ was purified by oligo(dT)-cellulose column chromatography (Aviv and Leder, 1972). Samples containing 5 μg of poly(A)$^+$ RNA were denatured in formamide, electrophoresed (1.2% agarose) in the presence of 6% formaldehyde (Maniatis *et al.* 1982) and blotted onto nitrocellulose filters as described (Thomas, 1980). Hybridization to 10^6–10^7 cts min^{-1} of labeled probes was carried out by incubation overnight at 42°C in a shaking waterbath in 50% formamide, 5×SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1×Denhardt's mixture, 1% SDS, 100 μg ml^{-1} of denatured salmon sperm DNA (0.1 ml cm^{-2} of filter). DNA fragments used for hybridization were labeled either by nick translation (Rigby *et al.* 1977) or by the random primer labeling technique developed by Feinberg and Vogelstein (1984) using [α-^{32}P]dATP with specific activities of 400 and 3000 Ci mmol^{-1}, respectively. The filters were subsequently washed three times with 5×SSC/0.2% SDS at 65°C, once with 2×SSC/0.2% SDS and once with 0.1×SSC/0.2% SDS at 25°C, and exposed for autoradiography, using Kodak XAR-5 films.

α-Factor internalization and extraction

Internalization and extraction of ^{35}S-labeled α-factor was assayed as described previously (Dulić and Riezman, 1989; Dulić *et al.* 1990). Cultures of *MAT***a** *bar1* cells were grown to exponential phase (1×10^7 to 2×10^7 cells ml^{-1}) in YPUAD medium at 24°C. The cells were harvested, washed with 50 mM potassium phosphate (pH 6) containing 1% BSA, and resuspended in ice-cold potassium phosphate buffer or YPUAD to 0.5×10^9 to 1.0×10^9 cells ml^{-1}. Binding of radioactive α-factor (approx. 10 Ci mmol^{-1}) was carried out with shaking for 1 h at 0°C (1×10^5 to 2×10^5 cts min^{-1} of α-factor per 10^9 cells). Unbound α-factor was washed away and the cells were resuspended in prewarmed phosphate buffer containing 2% glucose and incubated with shaking at 37°C. At the indicated times, duplicate samples of 100 μl were removed and diluted into 15–20 ml of either ice-cold pH 6 buffer to determine total cell-associated radioactivity, or the same volume of 50 mM sodium citrate–HCl, pH 1.15 (pH 1 buffer), to remove surface-bound α-factor, thus giving a measure of pheromone internalization. The latter samples were kept on ice for a minimum of 15 min to permit dissociation of externally bound α-factor by the pH 1 buffer. The cells were then collected on presoaked (1% BSA) filters (Millipore type HA, 0.45 μm pore size) mounted on a multifilter apparatus with a vacuum pump and washed three times with 5 ml of appropriate ice-cold buffer. Cells were eluted from the filters with 1 ml of pH 6 buffer, and pelleted and cell-associated α-factor was extracted in acidified methanol as described (Dulić and Riezman, 1989). The extracted radioactivity was applied directly onto 2 mm thick preparative silica gel 60 plates (Merck) and developed in a chamber saturated with *n*-butanol:propionic acid:water (50:25:35, by vol.). The plates were dried, sprayed with EN^{3}HANCE (New England Nuclear) and visualized by fluorography using Kodak XAR-5 films.

Pheromone response assays

G_1 arrest and morphological changes. Assays for cell-cycle

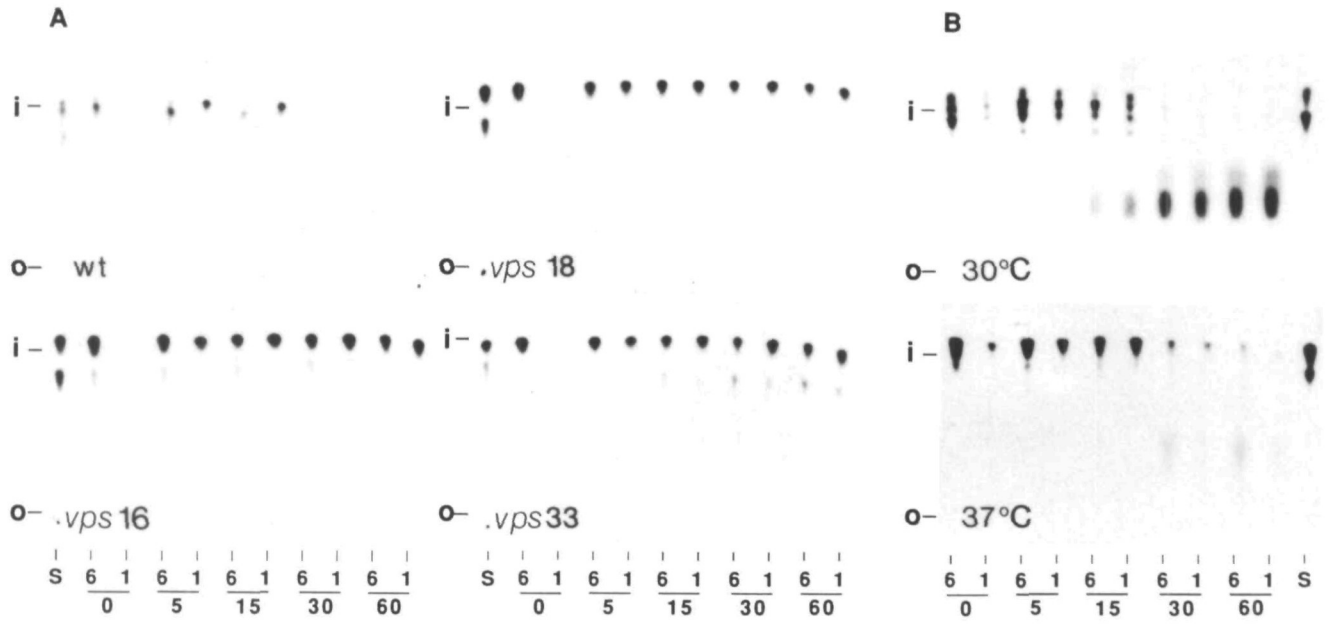

Fig. 1. Analysis of internalized α-factor in class C *vps* mutants and wild type (wt) cells. Cells were incubated with the ^{35}S-labeled α-factor (2×10^5 cts min^{-1} per 1×10^9 cells) for 1 h at 0°C, free radioactivity was washed away and the cells were warmed up to 37°C unless indicated otherwise. At indicated times (0–60 min), samples were removed and washed with the buffer at pH 6.0 (6), to determine total cell-associated radioactivity, or at pH 1.15 (1), to determine the internalized α-factor. Cell-associated radioactivity was then extracted, as described in Materials and methods, and analyzed by thin-layer chromatography (TLC) and autoradiography. (A) Extraction of cell-associated α-factor from the parental wild type cells (SEY6211-bar1), *vps16* (SEY1490-bar1), *vps18* (SEY1477-bar1) and *vps33* cells (SEY15194-bar1). (B) Extraction of cell-associated α-factor from wild type cells (RH144-3D) at 30°C and 37°C. i and o denote positions of the intact α-factor and origin of chromatography, respectively. As a standard (S), a sample of radioactive α-factor was used.

arrest and shmoo formation were performed using the microtiter dish assay (Ciejek and Thorner, 1979). The strains were grown at 24°C in YPUAD until mid-exponential phase. Cells were washed with YPUAD and incubated at 24°C for 5 h in 200 μl of YPUAD containing varying concentrations of α-factor at a cell density of 2×10^6 cells ml^{-1}. When pheromone response was scored at 37°C, the cells were gradually warmed to 37°C and incubated for 10 min at this temperature prior to addition of α-factor. The experiments were terminated by chilling the cells on ice. Cells were counted in a hemocytometer and cell morphology was scored. All buds larger than approximately 50% of the mother-cell size were counted as cells. Shmoos were identified as cells that show projection-like morphological changes and are unbudded (see Fig. 2, below).

Induction of STE2 *and* FUS1 *mRNA.* For the experiments monitoring the early pheromone response, wild type and *end1* cells were grown overnight to mid-exponential phase (2×10^7 cells ml^{-1}), washed once in YPUAD, and resuspended to 1×10^7 to 2×10^7 cells ml^{-1} in the indicated α-factor concentrations. Cells were incubated with shaking at 24°C for 30 min, harvested by centrifugation, chilled on ice, and poly(A)$^+$ RNA was extracted and electrophoresed as described above. Quantitation of the visualized transcripts was performed by densitometric scanning using the computing densitometer 300A (Molecular Dynamics, Sunnyvale, CA). The measurements were performed using the autoradiograms presented on the upper part of each panel, where all the bands were within the linear portion of the film's dose–response curve. The lower part of each panel (longer exposure) is shown for better visualization of the transcripts induced by low α-factor concentration. Results obtained upon quantitation of these bands corresponded to those obtained by using the short-exposure autoradiogram.

Pheromone induction of FUS1–lacZ protein. Wild-type and *end1* cells (as above) harboring a centromere plasmid that carries a *FUS1–lacZ* gene fusion (pSB231; Trueheart *et al.* 1987) were grown to mid-logarithmic phase in SD medium without uracil and then incubated for 2 h in the YPUAD medium. The cells were harvested, resuspended to 10^6 cells ml^{-1} in 10 ml of YPUAD, and warmed to the desired temperature. After 10 min, varying amounts of α-factor were added to each sample and the cells were incubated by shaking in a water bath for 1 h. Cells were then harvested (4°C), resuspended in the ice-cold lysis buffer (0.1 M sodium phosphate, pH 7.0, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol), and lysed by repeated vortexing in the presence of glass beads and 2 mM PMSF (phenylmethylsulfonyl fluoride). The supernatant was assayed in lysis buffer at 37°C for β-galactosidase activity using 2.5×10^{-4} M 4-methylumbelliferyl β-D-galactoside as a substrate (Leaback and Walker, 1961). Protein concentration was determined for each sample using the Pierce BCA protein assay reagent (Redinbaugh and Turley, 1986).

Quantitative mating assays

Mating efficiency of *end1* and wild type cells was assayed by two different quantitative tests (Hartwell, 1980; Sprague and Herskowitz, 1981). Two sets of isogenic strains were used that originated from the dissection of tetrads from the heterozygous diploid (RH444) carrying one copy of the disrupted *END1* gene (Dulić and Riezman, 1989). To allow for selection of diploids, appropriate haploid strains were rendered *trp1* by transformation with a DNA fragment that carries a *TRP1* coding region disrupted with the *URA3* gene. The strains used were RH144-3A, -3B, -3C, -3D, -3A-trp, -3B-trp, -3C-trp and -3D-trp.

Results

Class C vps *mutants internalize but do not degrade α-factor*

On the basis of previous data published by Chvatchko *et al.* (1986), it was proposed that class C *vps* mutants might be defective for endocytosis (Robinson *et al.* 1988). We have analysed internalization and degradation of radiolabeled α-factor in *vps16*, *vps18* and *vps33* cells. The increase in cell-associated and extractable radioactivity after an acidic wash (pH 1) demonstrates that α-factor was internalized in all cases (Fig. 1A) but it is degraded only in wild

type cells. The relatively small amounts of radioactivity observed at the later time points in the wild type reflect a poor yield of extraction of radioactivity rather than a low internalization efficiency. This occurs frequently at 37°C, but not at other temperatures such as 24 and 30°C (Fig. 1B; V. Dulić, Ph D thesis, University of Lausanne, 1989). We performed the experiments at 37°C because the nature of the mutations in *vps16*, *18* and *33* is not known. As the mutants do not grow at 37°C, their phenotype could be more severe at this temperature. These findings clearly show that, like *end1* (Dulić and Riezman, 1989), the class C *vps* mutants internalize but do not degrade radiolabeled α-factor.

Late response to α-factor and mating

It was reported earlier that the *end1* mutant does not form projections (shmoo) even at very high α-factor concentrations (Chvatchko *et al.* 1986). To examine this phenotype in more detail, we measured the late pheromone response by scoring for pheromone-induced shmoo formation and cell cycle arrest following exposure to various α-factor concentrations. The resulting dose–response curves (Fig. 2A and B) show that irrespective of the genetic background, original and null *end1* mutants require at least a tenfold higher concentration of α-factor than isogenic wild type cells to arrest in the cell cycle and shmoo. At low pheromone concentration (1×10^{-8} M for *bar1* cells) wild type cells undergo dramatic morphological changes featuring large and extended cells (Moore, 1984; see Fig. 3E). This stage seems to be missing in *end1* cells. Moreover, much higher pheromone concentrations are required to induce morphological change in most of the *end1* cells. Only 60–70% of the mutants have recognizable projections after incubation at 24°C for 5 h with 100 times higher α-factor concentration than necessary for a complete response of isogenic wild type cells. In addition to this pheromone insensitivity, *end1* shmoos exhibit a different, less-pronounced projection morphology and, under conditions where most of the wild type cells have more than one projection, very few *end1* cells do (Fig. 3K,L). When tested for shmoo formation at 37°C, *end1* cells are almost completely deficient in projection formation and cell cycle arrest (Fig. 2A). This is not due to extracellular degradation or inactivation of the α-factor, because when cells that have been incubated at 37°C are returned to 24°C shmoos form at their original efficiency without further addition of α-factor (data not shown).

Chvatchko *et al.* (1986) reported that even at concentrations as high as 5×10^{-5} M α-factor, *end1* cells do not form shmoos. We repeated these experiments with the identical strains (Fig. 4A) and always found a response at higher α-factor concentrations. We have no clear explanation for this discrepancy. One possibility is that *end1* shmoos were overlooked because they are less pronounced than wild type shmoos (see Fig. 3J,L,M).

The other class C *vps* mutants exhibit the same defects in α-factor-induced projection formation as those described for *end1* cells (Fig. 2C). They all require approximately 10 times more α-factor than the isogenic wild type to induce a similar percentage of shmoos. Because all of these strains both fail to degrade internalized α-factor and contain no functional vacuole, as a control we checked pheromone response in a *pep4* strain that also fails to degrade internalized α-factor but does contain a vacuole. We found that response of *pep4* cells to α-factor is indistinguishable from an isogenic wild type strain (Figs 2C and 3E,I).

Since binding assays and Scatchard analysis show that

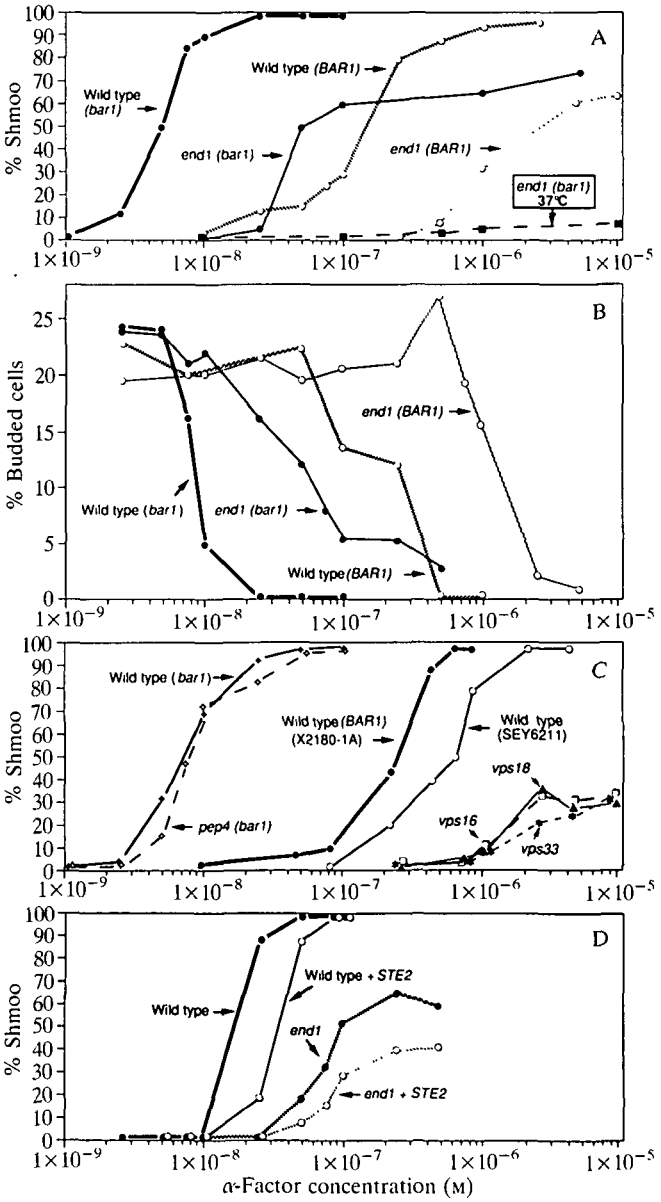

Fig. 2. Late pheromone response. (A, C and D) Dose–response curves for α-factor-induced projection formation in wild type and mutant cells. Assays were performed in microtiter plates at 2×10^6 cells ml^{-1} in 200 μl of YPUAD, 24°C, and various concentrations of α-factor. After 5 h, the plates were placed on ice to stop cell growth, and the cells were counted and their morphology scored in a hemocytometer. All buds larger than 50% of mother cell size were counted as cells. Unbudded cells with one or more projections were scored as shmoos (cf. Fig. 3). (B) Dose–response curves for α-factor-induced cell division arrest. Experimental conditions were as described above except that the cells were incubated for 2 h at 24°C. The strains are labeled as follows: (A) wild type, *bar1*=RH144-3D; *end1*, *bar1*=RH144-3B; wild type, *BAR1*=X2180-1A; and *end1*, *BAR1*=RH36-4 (X2180-1A genetic background); (B) RH144-3D; RH144-3B; wild type, *BAR1*=RH151-7B; *end1*, *BAR1*=RH151-7Be; (C) wild type, *bar1*=RH144-3D; *pep4*, *bar1*=RH732; wild type, *BAR1*=X2180-1A; SEY6211=parental strain for *vps* mutants; *vps16*=SEY1490; *vps18*=SEY1477; *vps33*=SEY15194; (D) wild type, RH144-3D-trp; *end1*, RH144-3B-trp. The *STE2* gene (encoding the α-factor receptor), where noted, was contained on CEN plasmid with the *TRP1* gene as a selectable marker. The control strains were transformed with the plasmid without the *STE2* gene.

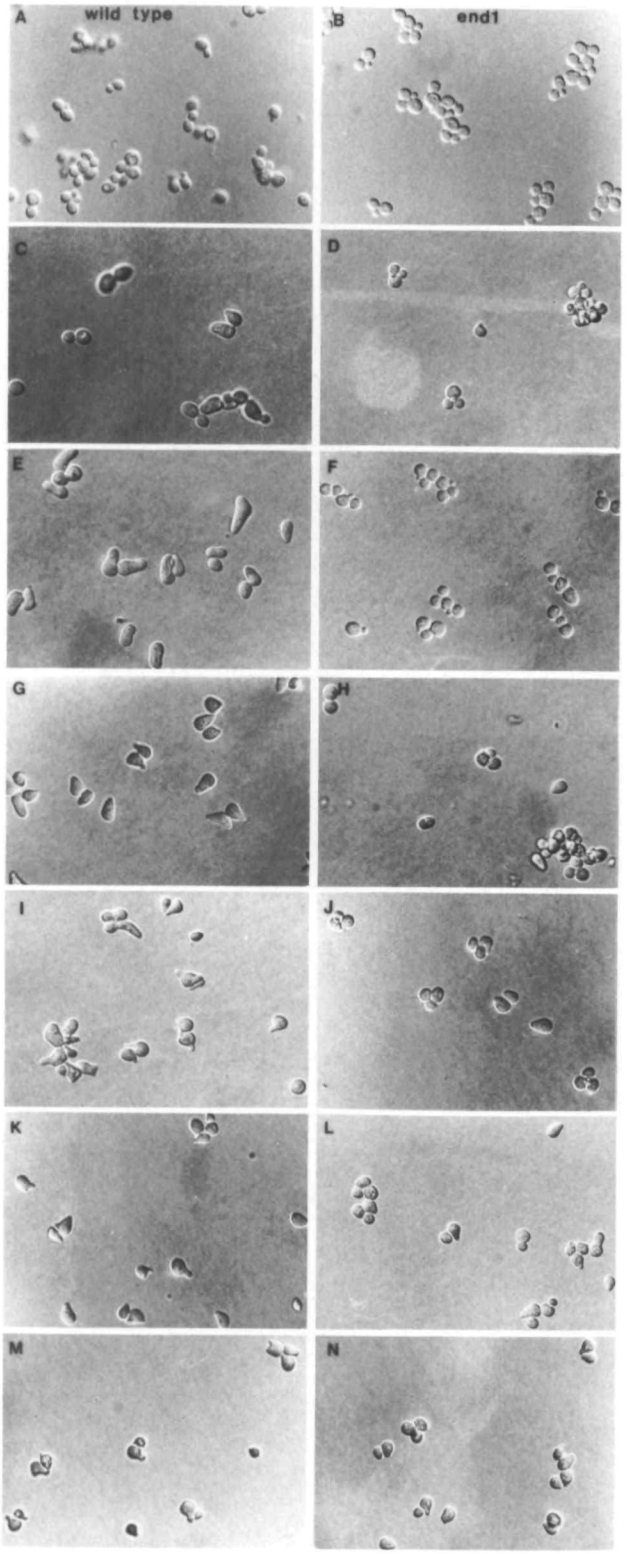

Fig. 3. α-Factor-induced projection formation in wild type, *pep4* and *end1* cells. The wild type or *pep4* (left side) and *end1* (right side) cells were incubated for 4.5 h at 24°C without (A,B) and in the presence of 2.5×10^{-9} M (C,D), 1×10^{-8} M (E,F), 2.5×10^{-8} M (G,H), 7.5×10^{-8} M (I,J), 1×10^{-7} M (K,L) and 1×10^{-6} M (M,N) of α-factor at cell concentration of 2×10^{6} cells ml^{-1}. The strains used were RH144-3D as a wild type, RH144-3B as *end1*, and RH732 (E and I) as an isogenic *pep4* strain. The photographs were taken using Nomarski optics.

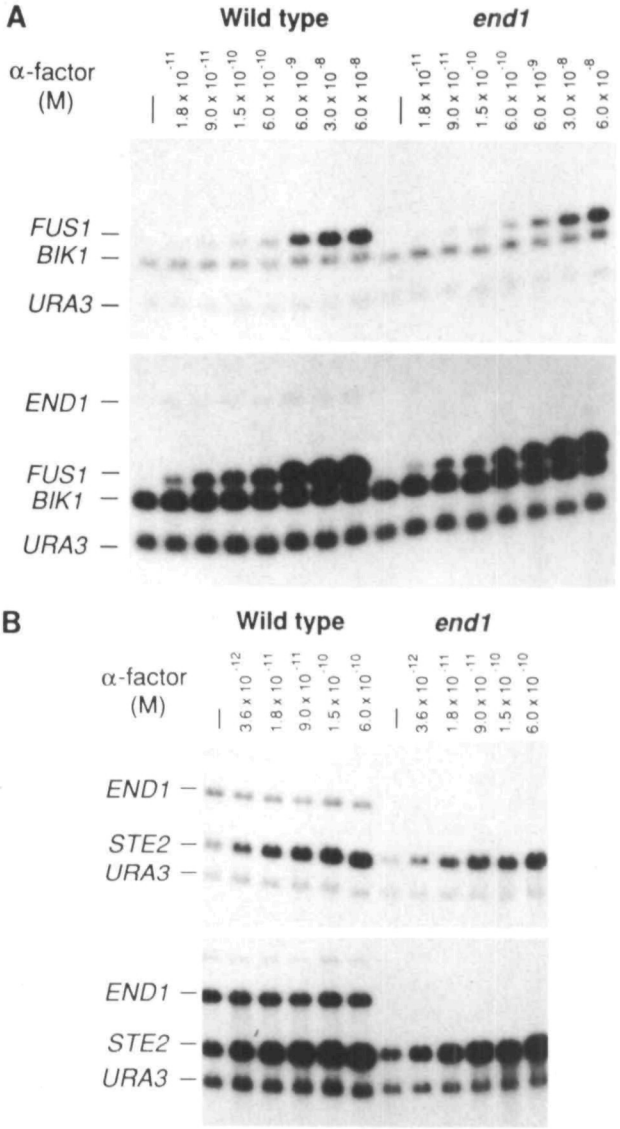

Fig. 4. Early pheromone response of *end1* and wild type cells: α-factor induced transcription of the *FUS1* and the *STE2* genes. Exponentially growing cells were harvested, washed with YPUAD and resuspended in 100 ml (2×10^{7} cells ml^{-1}) of the same medium. The cultures were incubated by shaking in the indicated concentrations of α-factor for 30 min at 24°C. Cells were then harvested (at 4°C) and poly(A)$^{+}$ RNA was extracted as described in Materials and methods. A 5 μg portion of poly(A)$^{+}$ RNA from each sample was separated on a formaldehyde-containing 1.2% agarose gel, transferred to a nitrocellulose filter, and hybridized with probes for the *END1*, *URA3*, *FUS1-BIK1* (A), *STE2* and *URA3* (B) genes. For better comparison, two different exposures for each experiment are shown (see also Fig. 5). Wild type (RH144-3D), and *end1* (RH144-3B) strains were used.

end1 cells have only 60–70% of wild type α-factor binding activity (Chvatchko *et al.* 1986; Dulić and Riezman, 1989; Y. Chvatchko, Ph D thesis, University of Lausanne, 1987), the impaired pheromone response of *end1* cells could be attributed solely to decreased receptor number. Therefore, we tested pheromone response by cells that harbor a CEN plasmid carrying the *STE2* gene. Both, *end1* and wild type cells bearing this plasmid show a 70% increase in surface α-factor receptors as detected by α-factor binding (data not

Table 2. *Quantitative efficiency of mating (%)*

Maters	Test A*	Test B†
Wild-type, α×wild-type, **a**	100	100
Wild-type, α×*end1*, **a**	31	85
end1, α×wild-type, **a**	36	44
end1, α×*end1*, **a**	4	43‡

Efficiency of mating was determined as described in Materials and methods.

*10^7 cells of each mater were mixed, pelleted and incubated in 4 ml YPUAD for 6 h at 25°C. The cells were diluted and plated onto selective plates at 30°C. Wild type mating efficiency was 0.5–1 % of the plated cells.

†A limited number of tester strain cells were plated onto selective plates containing a lawn of the opposite mating type strain. Wild type efficiency was 25–40 %.

‡The colonies were very heterogeneous in size.

shown). This increased α-factor binding capacity failed to render *end1* and wild type cells more sensitive to the pheromone, and in fact made them even less sensitive (Fig. 2D).

The deficiency in pheromone response of *end1* is also reflected in its mating efficiency; however, the magnitude of this defect depends on the type of mating test. We performed two different quantitative mating tests. In the first, **a** and α cells were mixed in YPUAD, pelleted, incubated submerged in YPUAD for 6 h at 25°C, and then plated onto plates selective for diploids (Hartwell, 1980). In the second, a lawn of **a** or α cells was plated onto selective plates and a known limiting quantity of cells of the opposite mating type was spread on top. Diploids were then scored to quantitate the percentage of cells that mated (Sprague and Herskowitz, 1981). By the first test, *end1* cells mate significantly less efficiently than wild type cells when either parent is an *end1* mutant, and even less efficiently when both parents are *end1* mutants (Table 2). By microscopic observation we found that in this mating test most wild type cells (vacuoles) showed pronounced projections while very few *end1* cells (no vacuoles) did. By the second test, the mating defect of *end1* mutants is much less severe. The difference in these two results could reflect the longer time available for mating in the second test. For this reason we checked the ability of *end1* to form shmoos at various α-factor concentrations (from 1×10^{-8} M to 1×10^{-5} M) over a 24 h period. At any particular time point, *end1* responds less well to α-factor than wild type. However, the number of shmoos increases with time and the projection morphology is more pronounced (in both wild type and *end1*), especially between 8 and 24 h (data not shown).

FUS1 *and* STE2 *gene induction by* end1

To elucidate whether the late pheromone defect of the *end1* mutant is accompanied by a defect in the signal transduction pathway, we studied the early pheromone response by following the steady-state level of expression of two genes known to be induced in haploid cells by mating factors. *STE2* encodes the α-factor receptor (Nakayama *et al.* 1985) while *FUS1* encodes a membrane protein that concentrates at the shmoo tip and is required for cell fusion during conjugation (Trueheart *et al.* 1987; Trueheart and Fink, 1989). *FUS1* mRNA is undetectable

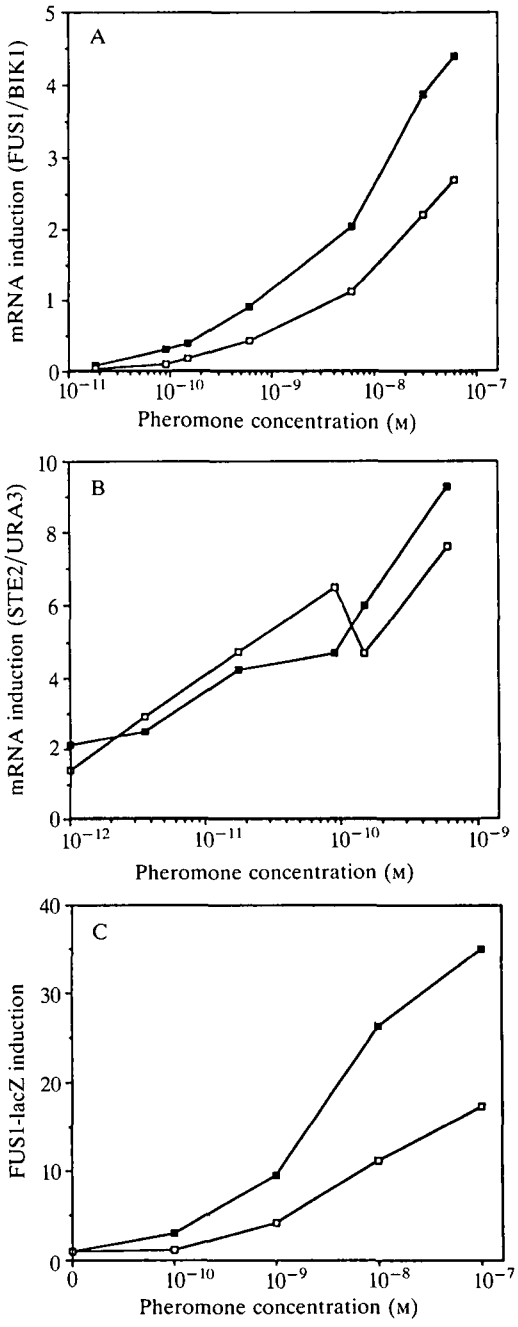

Fig. 5. Early pheromone response: quantitative analysis of the α-factor induction of *FUS1* and *STE2* in *end1* (□) and wild type (■) cells. The whole area of each band was quantitated by volume integration using the computing densitometer 300A (Molecular Dynamics, Sunnyvale, CA) and the results from integration of bands belonging to the *FUS1*, *BIK1*, *STE2* and *URA3* transcripts are presented. (A) The results obtained by scanning the autoradiogram from Fig. 4A. (B) The results presenting average values obtained by scanning two autoradiograms (one of them is shown in Fig. 4B). (C) α-Factor-mediated induction of the FUS1–lacZ fusion protein in *end1* and wild type cells after 1 h incubation in the presence of various concentrations of the pheromone at 37°C. The extent of induction was monitored by measuring the steady-state levels of β-galactosidase activity in cell lysates using 4-methylumbelliferyl β-D-galactoside as substrate (see Materials and methods). Each point on the graph is the average of three independent measurements. Fluorescence (excitation and emission wavelengths were 364 and 448 nm, respectively) with no α-factor added was taken as a standard of one arbitrary unit (ordinate) and all fluorescence values were normalized to this standard. The cells RH144-3B (*end1*) and RH144-3D (wild type) harbored the *URA3*-centromere plasmid pSB231 containing the *FUS1–lacZ* gene fusion (Trueheart *et al.* 1987).

in the absence of pheromone but is rapidly and strongly (40-fold) induced by its presence (McCaffrey *et al.* 1987).

Wild-type and *end1* cells grown to mid-log phase were incubated with various concentrations of α-factor for 30 min at 24°C to induce a maximal amount of the *FUS1* transcript. Poly(A)$^+$ RNA was prepared as described in Materials and methods. Northern blotting analysis of *STE2* and *FUS1* transcripts is shown in Fig. 4. The *BIK1* (a gene located near *FUS1*; cf. Trueheart *et al.* 1987), *URA3* and *END1* transcripts, not induced by mating factors, are used as controls. Quantitation of these transcripts (Fig. 5) show that: (1) the induction of the *FUS1* mRNA in *end1* cells requires a tenfold higher α-factor concentration than for wild type cells (Fig. 5A); (2) the level of induction of *STE2* mRNA is similar in both strains (Fig. 5B); (3) in both strains, the *STE2* mRNA is induced at apparently lower α-factor concentrations than the *FUS1* mRNA; (4) the levels of *STE2* message in cells not exposed to α-factor were lower for the *end1* mutant compared to wild type, suggesting that the lower receptor number in *end1* cells results directly from the steady-state mRNA level rather than from translational or post-translational control.

To test whether the defect in *FUS1* induction is more pronounced at 37°C, as is the morphological change, we followed α-factor-mediated induction of a *FUS1–lacZ* fusion protein (carried by plasmid pSB231) in wild type and *end1* cells. After 10 min preincubation at 37°C followed by 1 h exposure to several α-factor concentrations at 37°C, we found dose–response curves for the induction of the FUS1–lacZ fusion protein that are similar to those found at 24°C for induction of *FUS1* mRNA (Fig. 5C). These experiments demonstrate that (1) *end1* cells are defective for *FUS1* induction; (2) the failure to form shmoos at 37°C is probably not solely due to a defect of FUS1 induction.

Discussion

We report here that all class C *vps* mutants that lack a functional vacuole internalize but fail to degrade α-factor. As discussed previously for the *end1* mutant (Dulić and Riezman, 1989), this could reflect either the lack of vacuolar proteolytic activity (as is the case for the *pep4* mutant), or a defect in membrane traffic from a prevacuolar compartment to the vacuole. Such a compartment along the endocytic pathway has recently been detected in yeast (Singer and Riezman, 1990). In mammalian cells, endocytic markers and precursors of lysosomal enzymes pass through common intracellular organelles (Gruenberg *et al.* 1989). If present in yeast, the class C *VPS* gene products could be essential to this common pathway. Thus it is possible that in class C *vps* mutants the internalized α-factor resides in a prevacuolar compartment, similar to one of the common intermediates found in mammalian cells (Gruenberg *et al.* 1989).

The *end1* mutant is also partially defective for mating. This defect is more pronounced in a quantitative mating test in which **a** and α cells are coincubated for a fixed time compared to one in which cells are spread together onto selective medium. The reason for this difference could be twofold. First, the longer incubation times in the second test could enable the poorly responding *end1* cells to respond. Second, under selective conditions the cells are starving and this could help the mutant cells arrest at G_1 in the cell cycle. Konopka *et al.* (1988) reported that although cells expressing truncated α-factor receptor are defective for projection formation, they form zygotes at near wild type efficiency. The authors propose that acute projections are not essential for mating, which is consistent with our findings. It is also possible that only one mating partner needs to shmoo for successful conjugation.

We also show here that, in addition to their defect in α-factor metabolism, all four class C *vps* mutants have a defect in pheromone response. Although they require only an approximately 10-fold higher concentration of α-factor to initiate shmoo formation, none of these mutants exhibits the same morphological changes that are observed for their wild type counterparts, nor are they capable of reaching a 95–100% response even at saturating α-factor concentrations. The difference in shape of the α-factor-induced shmoos could reflect a less pronounced response in the mutant or could be explained by a possible role of the vacuole in carrying out changes in cell morphology.

The response defect of the *end1* mutant is most likely attributable to a partial defect in signal transduction. Microscopic observations of the wild type (vacuole) and *end1* (no vacuole) cells subjected to mating assay showed that only wild-type cells form projections normally ruling out extracellular inactivation of pheromone. The lower number of α-factor receptors on *end1* cells (60–70% of wild type) cannot account for this defect because cells carrying an additional copy of the *STE2* gene bind more α-factor and are less sensitive to pheromone. The fact that both *end1* and wild type cells respond slightly less well to α-factor when they carry an extra copy of the *STE2* gene suggests that the amount of cell-surface receptor has been optimized for the most sensitive response. α-Factor receptor could be optimized with respect to the amount of G-protein or other components of the signal transduction pathway.

As measures of the efficiency of the signal transduction pathway, we studied the α-factor-induced expression of the *STE2* and *FUS1* genes. These are good markers for this pathway because the induction of both mRNAs is rapid and requires no protein synthesis (Bender and Sprague, 1986; McCaffrey *et al.* 1987). We have found that the *end1* mutation does not impair induction of *STE2*, but does impair the induction of *FUS*1. A trivial explanation for the poor induction of *FUS1* mRNA in *end1* cells is that the induction is simply slower than normal. This is not satisfactory for several reasons. First, *STE2* mRNA is induced as rapidly in *end1* cells as in wild type cells. Second, *end1* cells can perform other tasks, such as α-factor uptake, as rapidly as wild type cells (Dulić and Riezman, 1989). Third, at high α-factor concentrations, *end1* cells respond as fast as wild type cells. The differences in induction of *STE2* and *FUS1* mRNA in wild type is intriguing. However, we cannot rule out that the *FUS1* RNA is induced at low pheromone concentration to an extent that is not detectable by our methods. If this difference is real, then there must be some qualitative or quantitative difference in the pheromone induction pathway leading to these two genes.

At 37°C, *end1* cells show a dramatic decrease in their ability to arrest in the cell cycle and shmoo in response to α-factor. In contrast, induction of the *FUS1* gene product is similar to that seen at 24°C. We propose two explanations. First, the response pathway may be similarly affected at 24°C and 37°C, but at the higher temperature *end1* cells cannot carry out the response. Alternatively, it could be that different branches of the signal transduction pathway

lead to gene induction, cell cycle arrest, and shmoo formation. In this model, the branch leading to cell cycle arrest and shmoo formation would be more dramatically affected. Recent evidence (Cross, 1988) supports the existence of such a branchpoint.

The striking phenotypic similarity of the class C *vps* mutants suggests that their primary defects are very similar. It is possible that all four gene products act directly on vacuole biogenesis and that the observed phenotypes result from the absence of the vacuole. As discussed above, those defects could result from the block in both endocytic and vacuolar biosynthesis pathways or on the vacuolar biosynthesis pathway alone. Alternatively, it is possible that all four genes act in concert or on subsequent steps of a common pathway required to produce a cofactor necessary for all of the processes that are defective in these mutants. Why are these mutants defective in certain aspects of the pheromone response? It is not due to a failure to internalize the pheromone. α-Factor is internalized normally by these mutants even though it is not degraded. α-Factor receptors lacking their cytoplasmic tail mediate a response to α-factor even though they are not internalized (Reneke *et al.* 1988). Pheromone degradation also should not affect response, because degradation normally occurs too late to account for the results with *FUS1* (Chvatchko *et al.* 1986; Dulić and Riezman, 1989) and because a *pep4* mutant responds identically to the isogenic wild type even though it does not degrade internalized α-factor (Singer and Riezman, 1990). Two other explanations are possible. The VPS proteins could play a direct role in the response pathway, or the impaired response could arise as a consequence of the other defects such as the lack of vacuole or alteration in vacuole biogenesis, which lead in turn to a disturbance of cellular homeostasis and perhaps changes in concentrations of cytoplasmic metabolites or the phosphorylation status of important regulatory proteins.

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References

Ammerer, G., Hunter, C. P., Rothman, J. H., Saari, G. C., Valls, L. A. and Stevens, T. H. (1986). *PEP4* gene of *Saccharomyces cerevisiae* encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. *Molec. cell. Biol.* **6**, 2490–2499.

Aviv, H. and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408–1412.

Banta, L. M., Robinson, J. S., Klionsky, D. J. and Emr, S. D (1988). Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting *J. Cell Biol.* **107**, 1369–1383.

Bender, A. and Sprague, G. F. (1986). Yeast peptide pheromones, a-factor and α-factor, activate a common response mechanism in their target cells. *Cell* **47**, 929–937.

Chattoo, B. B., Sherman, F., Atubalis, D. A., Fiellstedt, T. A., Mehnert, D. and Ogur, M. (1979). Selection of *lys2* mutants of the yeast *S. cerevisiae* by utilization of α-aminoadipate. *Genetics* **93**, 51–65.

Chvatchko, Y., Howald, I and Riezman, H. (1986). Two yeast mutants defective in endocytosis are defective in pheromone response. *Cell* **46**, 355–364.

Ciejek, E. and Thorner, J (1979). Recovery of *Saccharomyces cerevisiae* a cells from G1 arrest by α factor pheromone requires endopeptidase action. *Cell* **18**, 623–635.

Cross, F. R. (1988). *DAF1*, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. *Molec. cell. Biol.* **8**, 4675–4684.

Cross, F., Hartwell, L. H., Jackson, C. and Konopka, J. B (1988). Conjugation in *Saccharomyces cerevisiae*. *A. Rev. Cell Biol.* **4**, 429–457.

Dietzel, C. and Kurjan, J. (1987). The yeast *SCG1* gene: a Gα-like protein implicated in the a- and α-factor response pathway. *Cell* **50**, 1001–1010.

Dixon, R. A. F., Kobilka, B. K., Stadler, D. J., Benovic, J L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M G , Lefkowitz, R. J. and Strader, C. D. (1986). Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature* **321**, 75–79.

Dulić, V., Egerton, M., Elguindi, I., Raths-Hooper, S , Singer, B. and Riezman, H. (1990). Yeast endocytosis assays. *Meth Enzym.* **194** (in press).

Dulić, V. and Riezman, H. (1989). Characterization of the *END1* gene required for vacuole biogenesis and gluconeogenic growth of budding yeast. *EMBO J.* **8**, 1349–1359.

Emr, S. D , Vassarotti, A., Garrett, J., Geller, B. L., Takeda, M. and Douglas, M. G. (1986). The amino terminus of the yeast F1-ATPase β subunit precursor functions as a mitochondrial import signal. *J. Cell Biol.* **102**, 523–533.

Feinberg, A. P. and Vogelstein, B. (1984). "A technique for radiolabeling DNA restriction fragments to high specific activity" Addendum. *Analyt. Biochem.* **137**, 266–267.

Gruenberg, J., Griffiths, G. and Howell, K. (1989). Characterization of the early endosome and putative endocytic carrier vesicles *in vivo* and with an assay of vesicle fusion *in vitro*. *J. Cell Biol.* **108**, 1310–1316.

Hagen, D. C., McCaffrey, G. and Sprague, G. F. (1986). Evidence the yeast *STE3* gene encodes a receptor for the peptide pheromone a factor: Gene sequence and implications for the structure of the presumed receptor. *Proc. natn. Acad. Sci. U.S.A.* **83**, 1418–1422

Hartwell, L. H. (1980). Mutants of *Saccharomyces cerevisiae* unresponsive to cell division control by polypeptide mating hormone. *J. Cell Biol.* **85**, 811–822.

Herskowitz, I. (1989). A regulatory hierarchy for cell specialization in yeast. *Nature* **342**, 749–757.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bact.* **153**, 163–168.

Jenness, D. D., Burkholder, A. C. and Hartwell, L H. (1983). Binding of α-factor pheromone to yeast a cells. chemical and genetic evidence for an α-factor receptor. *Cell* **35**, 521–529

Jenness, D D. and Spatrick, P. (1986). Down regulation of the α-factor pheromone receptor in *Saccharomyces cerevisiae*. *Cell* **46**, 345–353.

Jensen, R., Sprague, G. F., Jr and Herskowitz, I. (1983). Regulation of yeast mating-type interconversion: feedback control of *HO* gene expression by mating-type locus. *Proc. natn. Acad. Sci U.S.A.* **80**, 3035–3039.

Jones, E. W. (1977). Proteinase mutants of *Saccharomyces cerevisiae*. *Genetics* **85**, 23–33

Jones, E. W., Zubenko, G. S. and Parker, R. R. (1982). Genetic properties of mutations at the *PEP4* locus in *Saccharomyces cerevisiae* *Genetics* **102**, 655–677

Konopka, J. B., Jenness, D. D and Hartwell, L. H. (1988). The C-terminus of the *Saccharomyces cerevisiae* α-pheromone receptor mediates an adaptive response to pheromone. *Cell* **54**, 609–620.

Leaback, D. H. and Walker, P. G. (1961). Studies on glucosaminidase· 4. The fluorimetric assay of *N*-acetyl-β-glucosaminidase. *Biochem. J.* **78**, 151–156.

MacKay, V. L., Welch, S. K , Insley, M. Y., Manney, T. R., Holly, J., Saari, G. C. and Parker, M. L. (1988). The *S. cerevisiae BAR1* gene encodes an exported protein with homology to pepsin. *Proc natn Acad. Sci U S.A* **85**, 55–59.

Maniatis, T., Fritch, E. F. and Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Matile, P. (1978). Biochemistry and functions of vacuoles *A. Rev. Pl. Physiol.* **29**, 193–213.

McCaffrey, G , Clay, F J., Kelsay, K. and Sprague, G F. (1987). Identification and regulation of a gene required for cell fusion during mating of the yeast *Saccharomyces cerevisiae*. *Molec. cell. Biol.* **7**, 2680–2690.

Miyajima, I., Nakafuku, M., Brenner, C., Miyajima, A., Kaibuchi, K., Arai, K., Kaziro, Y. and Matsumoto, K. (1987). *GPA1*, a haploid-specific essential gene, encodes a yeast homolog of mammalian G

protein which may be involved in mating factor signal transduction. *Cell* **50**, 1011–1019

Moore, S. (1984). Yeast cells recover from mating pheromone α-factor-induced division arrest by desensitization in the absence of α-factor destruction. *J. biol. Chem.* **259**, 1004–1010.

Nakayama, N., Miyajima, A. and Arai, K. (1985). Nucleotide sequence of *STE2* and *STE3*, cell type-specific sterile genes from *Saccharomyces cerevisiae*. *EMBO J.* **4**, 2643–2648.

Redinbaugh, M. G. and Turley, R. B. (1986). Adaptation of the bicinchonic acid protein assay for use with microtiter plates and sucrose gradient fractions. *Analyt. Biochem.* **153**, 267–271.

Reneke, J. E., Blumer, K J , Courchesne, W. E. and Thorner, J. (1988). The carboxy-terminal of the yeast α-factor receptor is a regulatory domain. *Cell* **55**, 221–234.

Rigby, P. W., Dieckmann, M., Rhodes, C. and Berg, P (1977). Labeling deoxyribonucleotide acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. molec. Biol.* **133**, 237–251.

Robinson, J. S., Klionsky, D. J., Banta, L. M. and Emr, S. E. (1988). Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Molec. cell. Biol.* **8**, 4936–4948.

Rothman, J. H., Howald, I. and Stevens, T. H. (1989). Characterization of genes required for protein sorting and vacuolar function in the yeast *Saccharomyces cerevisiae*. *EMBO J.* **8**, 2057–2065.

Rothstein, R. J. (1983). One-step gene disruption in yeast. *Meth. Enzym* **101**, 202–211.

Singer, B. and Riezman, H. (1990). Detection of an intermediate compartment involved in transport of α-factor to the vacuole in yeast. *J. Cell Biol.* **110**, 1911–1922.

Sprague, G. F. and Herskowitz, I. (1981). Control of yeast cell type by the mating type locus. *J. molec. Biol.* **153**, 305–321.

Thomas, P. S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. natn Acad. Sci. U.S.A.* **77**, 5201–5205.

Trueheart, J , Boeke, J. D. and Fink, G. R. (1987). Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. *Molec. cell. Biol.* **7**, 2316–2328.

Trueheart, J. and Fink, G. R. (1989). The yeast cell fusion protein FUS1 is O-glycosylated and spans the plasma membrane. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9916–9920.

Whiteway, M., Hougan, L., Dignard, D., Thomas, D. Y., Bell, L., Saari, G. C., Grant, F. J., O'Hara, P. and MacKay, V. L. (1989). The *STE4* and *STE18* genes of yeast encode potential β and γ subunits of the mating factor receptor-coupled G protein. *Cell* **56**, 467–477

Wiemken, A., Schellenberg, M. and Urech, K. (1979). Vacuoles: the sole compartments of digestive enzymes in yeast (*S. cerevisiae*)? *Arch. Microbiol* **123**, 23–35.

Westenberg, B., Boller, T. and Wiemken, A. (1989). Lack of arginine- and polyphosphate-storage pools in a vacuole-deficient mutant (*end1*) of *Saccharomyces cerevisiae*. *FEBS Lett.* **254**, 133–136.

Woolford, C. A., Daniels, L. B., Park, F. J., Jones, E. W., Van Arsdell, J. N. and Innis, M. A (1986). The *PEP4* gene encodes an aspartyl protease implicated in the postranslational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. *Molec cell. Biol.* **6**, 2500–2510

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