Detection of an Intermediate Compartment Involved in Transport of α-Factor from the Plasma Membrane to the Vacuole in Yeast

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Abstract. α-Factor, one of the mating pheromones of *Saccharomyces cerevisiae*, binds specifically to a receptor on the plasma membrane of a cells, is internalized and delivered to the vacuole, where it is degraded. At 15°C the rate of pheromone uptake is only slightly affected while delivery to the vacuole is markedly slowed down. A transport intermediate carrying α-factor to the vacuole can be reversibly trapped by treatment with the metabolic inhibitors, NaN₃ and NaF. This intermediate(s) is distinct from the vacuole and the plasma membrane as judged by differential and density gradient centrifugation analysis. We present evidence that the α-factor is protected from protease digestion by a detergent-sensitive structure, suggesting that the pheromone resides within a vesicular compartment. We propose that this intermediate(s) represents an endocytic or prevacuolar compartment(s) involved in vesicular traffic from the plasma membrane to the vacuole.

THE budding yeast *Saccharomyces cerevisiae* is a unicellular organism that requires the action of peptide pheromones for the conjugation of haploid a and α cells (Hartwell, 1973; Sprague et al., 1983; Kurjan, 1985). a cells respond to α-factor pheromone that is secreted by α cells; α cells respond to a-factor pheromone secreted by a cells. The reciprocal action of these two diffusable peptide hormones mediates a series of events that culminate in the production of a/α diploid cells: transcription of several genes is induced (Hagen and Sprague, 1984; Stetler and Thorner, 1984) and mating-specific cell surface agglutinins are produced (Fehrenbacher et al., 1978). Cell growth is arrested in the G₁ phase of the cell cycle (Hartwell, 1973), and cell and nuclear fusion are coordinated (Trueheart et al., 1987; McCaffrey et al., 1987; Rose et al., 1986). An additional response to the mating factors includes the induction of a morphological change, called the "shmoo." It is characterized by a local elongation of the yeast cell, providing the fusion site during zygote formation (reviewed by Thorner, 1981; and Sprague et al., 1983).

Signals to initiate the mating program in yeast are transduced through distinct cell surface receptors, specific for each mating factor. The α-factor receptor, encoded by the *STE2* gene (Jenness et al., 1983, 1986; Blumer et al., 1988), is a 431 residue polypeptide that contains seven putative membrane-spanning domains (Burkholder and Hartwell, 1985; Nakayama et al., 1985). This topological motif is shared by the a-factor receptor (encoded by the *STE3* gene), although there is no apparent sequence homology between the two proteins (Nakayama et al., 1985). Jenness and Spatrick (1986) provided evidence that the α-factor receptor

is subject to downregulation. This event is accompanied by internalization of α-factor. Independently, Chvatchko et al. (1986) demonstrated that the pheromone is internalized by a cells in a time-, temperature-, and energy-dependent fashion and furthermore that it is degraded, most likely in the vacuole (Chvatchko, 1987; Dulić, 1989). These findings led to the hypothesis that α-factor is internalized by receptor-mediated endocytosis. In mammalian cells several polypeptide hormones like insulin and epidermal growth factor are taken up by receptor-mediated endocytosis via clathrin coated pits (Carpenter and Cohen, 1976; Gavin et al., 1974). The β-adrenergic receptor, which has a striking structural similarity to the α-factor receptor, has also been demonstrated to be sequestered, although the pathway of this internalization is less understood (Sibley and Lefkowitz, 1985).

In this study we have followed the uptake and degradation of ³⁵S-α-factor to investigate the endocytic pathway in yeast. This system has the advantage of being physiologically relevant in contrast to systems using nonphysiological markers (Riezman, 1985; Makarow, 1985*a b*; Makarow and Nevalainen, 1987). Although downregulation of receptor sites and transport of the pheromone presumably to the vacuole are consistent with the hypothesis of receptor-mediated endocytosis, a direct demonstration of a vesicular uptake mechanism is clearly desirable. To facilitate the demonstration and isolation of a potential vesicular intermediate involved in α-factor uptake and transport, we have made use of a low temperature incubation and metabolic inhibitors. These manipulations have been used successfully in mammalian cells to block transport from endosomes to lysosomes (Dunn et al., 1980; Marsh et al., 1983; Griffiths et

al., 1988; Davey et al., 1985; Gruenberg and Howell, 1986). We show that transport of α-factor to the vacuole, where degradation most likely takes place, is significantly slowed down at a low temperature and can be further blocked in the presence of metabolic inhibitors. Applying these conditions, we can trap α-factor within a membrane-sealed compartment(s) that is distinct from the vacuole and the plasma membrane. Therefore, we provide the first evidence for a membranous intermediate compartment(s) containing α-factor, a hallmark of endocytosis.

Materials and Methods

Strains, Media, and Reagents

The strains of *S. cerevisiae* used in these experiments were RH144-3D (Mata, his4, leu2, ura3, bar1-1) and RH732 (Mata, his4, leu2, ura3, lys2, bar1-1, pep4::URA3). This strain was derived from RH448 (Mata, his4, leu2, ura3, lys2, bar1-1) by a one-step gene disruption using the plasmid pTS15 (kindly provided by T. Stevens, University of Oregon, Eugene, OR). RH449 (Matα, his4, leu2, ura3, lys2, bar1-1) was used for α-factor production. All yeast strains were grown in complete medium (2% yeast extract, 2% peptone [both Gibco Ltd., Paisley, Great Britain], 40 μg/ml each adenine [Sigma Chemical Co., St. Louis, MO] and uracil [E. Merck, Darmstadt, Federal Republic of Germany], and 2% glucose) to exponential phase (2 × 10⁷ cells/ml) at 30°C on a rotary shaker. Lyticase was prepared as described by Scott and Schekman (1980). ³⁵S-sulfate (43 Ci/mg) was obtained from Du Pont de Nemours International S. A. (Regensdorf, Switzerland). Proteinase K was from Merck and BSA was from Sigma Chemical Co. Silica gel 60 plates (analytical and preparative) were purchased from Merck, and solvents were either from Fluka AG (Buchs, Switzerland) or Merck. Nycodenz was obtained from Nyegaard Co. (Oslo, Norway) and BCA protein assay reagent from Pierce Chemical Co. (Rockford, IL). Chemicals for SDS-PAGE were purchased from Bio-Rad (Richmond, CA), nitrocellulose filters were from Millipore Continental Water Systems (Bedford, MA), and Ponceau S was obtained from Serva (Heidelberg, FRG). ENTENSIFY and EN³HANCE spray were from Du Pont de Nemours International S. A. NaN₃ was purchased from Merck and NaF was from Sigma Chemical Co. Buffers, Triton X-100, and quinacrine were from Sigma Chemical Co. DEAE-dextran was obtained from Pharmacia (Uppsala, Sweden) and Amberlite ion exchange resin, type CG-50 (mesh size 100-200) was from Serva.

Production of ³⁵S-α-Factor

³⁵S-labeled α-factor was synthesized and isolated as previously described by Dulić and Riezman (1989). In brief, RH449 cells were transformed with the plasmid pDA6300 (kindly provided by J. Thorner, University of California, Berkeley, CA), a 2-μ based plasmid containing the *MFα1*, *STE13*, and *LEU2* genes. Transformants, showing at least 10-fold overproduction of α-factor (monitored by halo assay) were used for biosynthetic labeling and α-factor was prepared from the culture supernatant by chromatography on CG-50 (Jenness et al., 1983). Fractions containing biologically active α-factor, as determined by either halo assay or shmoo assay, were pooled, dried, and further purified using HPLC (μ BONDAPAK™ C18 column, 300 × 3.9 mm ID, 10-μm particle size; Millipore). α-Factor was eluted under isocratic conditions with 29.6% acetonitrile, 0.025% trifluoroacetic acid. Fractions were analyzed by TLC using the solvent system butanol/propionic acid/water (100:50:70, vol/vol/vol) with chemically synthesized α-factor as a standard on silica gel 60 plates. Purified ³⁵S-α-factor was dried and resuspended in 0.01 N HCl, 1 mM DTT, 0.2 mM EDTA and stored in small aliquots in liquid nitrogen. The specific radioactivity of ³⁵S-α-factor ranged from 5 to 10 Ci/mmol of active pheromone.

α-Factor Uptake and Degradation Assays

Pheromone internalization and degradation assays were carried out as described by Dulić and Riezman (1989). Uptake assays were performed at 5, 10, 15, 19, or 30°C, using the strains RH144-3D and RH732. When α-factor degradation was analyzed in the presence of metabolic poisons, NaN₃ and NaF were added to 20 mM each (final concentration) after allowing uptake for various times at 15°C as indicated in the figures. At the same time the

cells were transferred to a 30°C waterbath. The radioactivity was extracted from cells and resolved by TLC after different times of internalization as indicated in the figures. To analyze the reversibility of the NaN₃ and NaF treatment, cells were incubated for 15 min at 15°C, NaN₃ and NaF were added, and the cells were shifted to 30°C. After 15 min the cells were harvested and resuspended in inhibitor-free medium, and uptake was continued at 30°C. When bafilomycin A₁ (kind gift of K. Altendorf, University of Osnabrück, Osnabrück, Germany) was used, a 6.6 mM stock was prepared in DMSO, and the drug (or DMSO alone) was added to 10 μM final concentration after allowing uptake to occur for 15 min at 15°C. Lower concentrations of bafilomycin A₁ were not effective in elevating vacuolar pH, as determined by quinacrine staining (see below). Upon addition of the drug the temperature was raised to 30°C. 10, 45, and 105 min after the addition of bafilomycin A₁ cells were stained with quinacrine as described by Banta et al. (1988) and immediately viewed by epifluorescence using a Zeiss Axiophot microscope. Intact and degraded α-factor were resolved by TLC (using preparative 2.2 mm silica-gel 60 plates) as described above, and fluorographs on preflashed films (Kodak XAR-5) were quantified by densitometric scanning on a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Cell Fractionation

For each fractionation experiment using differential centrifugation analysis ∼5 × 10⁹ RH732 cells were harvested from an exponentially growing culture, resuspended in complete medium to 1 × 10⁹ cells/ml, and chilled on ice. ³⁵S-α-factor (6 × 10⁵ cpm) was added and allowed to bind for 1 h on ice. The cells were resuspended in the same volume of ice-cold, 15 or 30°C complete medium and were further incubated for 30, 20, or 30 min, respectively. After this incubation the cells were diluted ∼10-fold with ice-cold sorbitol medium (0.6 M sorbitol, 5 mM 2-amino-2methyl-1,3-propanediole-Pipes, pH 6.8) and harvested. The cells were washed with ice-cold sorbitol medium, resuspended in 20 ml of 0.14 M cysteamine-HCl, 0.6 M sorbitol, 25 mM 2-amino-2methyl-1,3-propanediole-Pipes, pH 6.8, 5 mM EDTA, 5 mM NaN₃, and 5 mM NaF and were incubated at 30°C for 20 min. After this pretreatment, the cells were diluted with ice-cold sorbitol medium, pelleted, and resuspended in 1 ml sorbitol medium, containing 5 mM NaN₃ and 5 mM NaF and ∼1 × 10⁴ U lyticase. The cells were converted into spheroplasts at 30°C for 1 h and centrifuged at 1,500 g at 4°C through a 0.6 M sucrose cushion, containing 5 mM 2-amino-2-methyl-1,3-propanediole-Pipes, pH 6.8. The pellet was resuspended with 4.5 ml ice-cold sorbitol medium and the spheroplasts were lysed by adding 90-135 μl of 10 mg/ml DEAE-dextran (in sorbitol medium) at 0°C and then warming up to 30°C for 5-7 min. The lysate was subjected to three successive centrifugation steps at 4°C. First, it was centrifuged at 3,500 g for 5 min, resulting in P1 and S1. The supernatant S1 was centrifuged at 10,000 g for 10 min (giving rise to P2 and S2). Finally the supernatant S2 was subjected to centrifugation at 100,000 g resulting in P3 and S3. The three pellets (P1, P2, and P3) were resuspended in ice-cold sorbitol medium and aliquots of each of the six fractions were counted for ³⁵S-α-factor or were TCA precipitated and resuspended in SDS gel sample buffer (50 mM Tris, pH 6.8, 2.5 mM EDTA, 2% [wt/vol] SDS, 5% [wt/vol] glycerol, 0.01% [wt/vol] bromphenol blue, and 2.5% [vol/vol] 2-mercaptoethanol). Two samples from each fraction (∼20 and 40 μg protein) were resolved on 7.5% polyacrylamide gels containing 8 M urea using the buffer system of Laemmli (1970). After electrophoresis, proteins were transferred to nitrocellulose, as described by Towbin et al. (1979). After transfer, the nitrocellulose filter was stained with Ponceau S and incubated for 1 h with blocking buffer (2.5% [wt/vol] dry fat milk, 0.2% [wt/vol] Triton X-100 in PBS). The filters were either treated with antiserum against the plasma membrane H⁺-ATPase (kindly provided by N. Nelson, Roche Institute of Molecular Biology, Nutley, NJ, diluted 1:2,000), against carboxypeptidase Y (CPY)[1] (diluted 1:500) or against hexokinase (kindly provided by G. Schatz, Biocenter, Basel, Switzerland; diluted 1:1,000). Incubation with the antiserum was carried out for 1 h with shaking at room temperature. The filters were washed four times with blocking buffer. After washing, the filters were incubated with 1-2 μCi of ¹²⁵I-protein A in blocking buffer for 1 h as described before. After air drying, the filters were subjected to fluorography at −70°C using intensifying screens. Fluorographs on preflashed films (Kodak XAR-5) were quantified by densitometric scanning on a TLC scanner (type II; Camag, Muttenz, Switzerland). Since the extent of cell lysis varied between individual experiments, it was necessary to correct the individual values for cell lysis in order to be able to compare the various conditions and different experiments. The

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1. *Abbreviation used in this paper*: CPY, carboxypeptidase Y.

determination of the extent of cell lysis was based on the distribution of cytoplasmic hexokinase after centrifugation at 3,500 g. From localization studies of hexokinase by immunocytochemistry (van Tuinen and Riezman, 1987), we calculated that ~95% of the total cellular hexokinase is in the cytoplasm and 5% is in nuclei. Although these 5% should pellet at 3,500 g when 100% of the cells are lysed, we neglected this small fraction in our calculations and considered the amount of hexokinase found in P1 to originate from unlysed cells. The fraction of unlysed cells was calculated as follows: fraction of unlysed cells = $P1_{hexokinase}/(P1_{hexokinase} + S1_{hexokinase})$. The average lysis in 18 experiments was 63 ± 21%. The amount of the other markers found in P1 was corrected for cell lysis by the following formula: P1 = $P1_{uncorrected}$ − (fraction of unlysed cells) × $(P1_{uncorrected} + S1)$. The total was obtained by addition of P1 and S1.

For subfractionation on density gradients ~5 × 10^{10} cells and 1.5–2.5 × 10^6 cpm ^{35}S-α-factor were used as the starting material. Uptake of ^{35}S-α-factor was carried out for 20 min at 15°C and the P3 fraction was obtained as described before except that the second differential centrifugation step was carried out at 7,500 g for 5 min in order to optimize the recovery of α-factor in the P3 pellet. This fraction was resuspended in 1.1 ml of 35% (wt/vol) Nycodenz (Nycodenz stock solution) and homogenized five times using a 1-ml tissue grinder (Wheaton Industries, Millville, NJ). The homogenate was transferred to a 13.2-ml centrifuge tube (Ultra-Clear; Beckman Instruments Inc., Palo Alto, CA) and a discontinuous gradient was prepared by overlaying 1 ml of homogenate with 2.5 ml each of 31, 27, 20, and 13% Nycodenz solution. These solutions were dilutions of the Nycodenz stock solution with sorbitol medium (so that the decreasing Nycodenz concentrations were accompanied by an increase in sorbitol and buffer concentration). The gradient was subjected to centrifugation at 4°C for 14–16 h at 100,000 g in a TST41.14 swinging bucket rotor (Kontron Instruments, Zürich, Switzerland). A total of 17 fractions (each ~650 μl) were collected from the top of the gradient by pumping 60% (wt/vol) sucrose into the bottom of the tube. A major part of the pellet formed during centrifugation was not collected. This probably explains why only 40% of the protein loaded onto the gradient was recovered. However, >95% of ^{35}S-α-factor was recovered. Aliquots from each fraction were analyzed for ^{35}S-α-factor and H$^+$-ATPase as described before. Protein concentration was assayed by the BCA procedure described in a company brochure by the manufacturer (Pierce Chemical Co.). The density of the fractions was determined by measuring the refractive index and reading the corresponding density from a standard curve. The standard curve was obtained from a mock gradient by determining the refractive index and the density of each fraction.
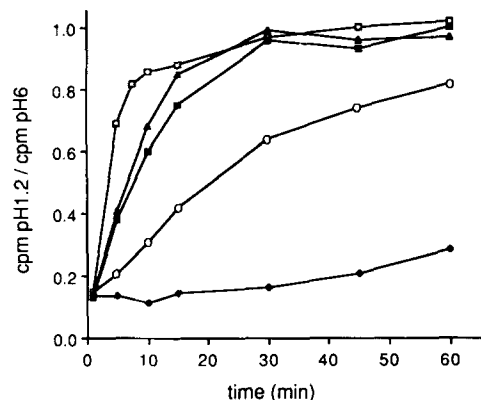

Figure 1. Effect of temperature on α-factor uptake. a cells (RH144-3D) were incubated with ^{35}S-α-factor (1 × 10^5 cpm/10^9 cells) for 1 h at 0°C as described in Materials and Methods. The cells were harvested and resuspended in prewarmed, glucose-containing, potassium phosphate buffer. Uptake was done at 5 (♦), 10 (○), 15 (■), 19 (▲), and 30°C (□). At the indicated times duplicates of 10^8 cells were washed with pH 1.2 buffer to determine internalized α-factor and with pH 6 buffer to determine cell-associated α-factor. After filtering the cells, the associated radioactivity was determined by liquid scintillation counting. Uptake is expressed as the ratio between internalized and total cell-bound radioactivity (total cell-bound radioactivity ranged from 5,500 to 8,500 cpm). The values obtained at 30, 19, 15, and 10°C are an average of at least three experiments. Uptake at 5°C was done once.

Table I. $t_{1/2}$ for α-Factor Uptake and α-Factor Degradation as a Function of Temperature

Temperature	$t_{1/2}$ uptake	$t_{1/2}$ degradation
°C	min	min
30	3.75	10
19	6.75	23
15	7.5	53
10	23	>120

^{35}S-α-factor uptake and degradation experiments were performed as described in the legends to Fig. 1 and Fig. 3. The $t_{1/2}$ for pheromone uptake was calculated from the initial rate of uptake for the curves shown in Fig. 1. The $t_{1/2}$ for α-factor degradation (disappearance of intact, internalized α-factor) was calculated in the following way. The amount of internalized, intact α-factor (pH 1.2 samples) was determined by densitometric scanning of fluorographs as shown in Fig. 3. After an initial increase in intact, internalizaed α-factor the data gave a linear decay curve on a logarithmic scale. The $t_{1/2}$ is the time necessary to degrade 50% of the remaining intact α-factor. The values given for 30 and 15°C are the means of three experiments, the $t_{1/2}$ for degradation at 19°C was calculated from two experiments, and at 10°C from one experiment. The radioactivity from the internalized, intact α-factor is generally recovered in the degradation products.
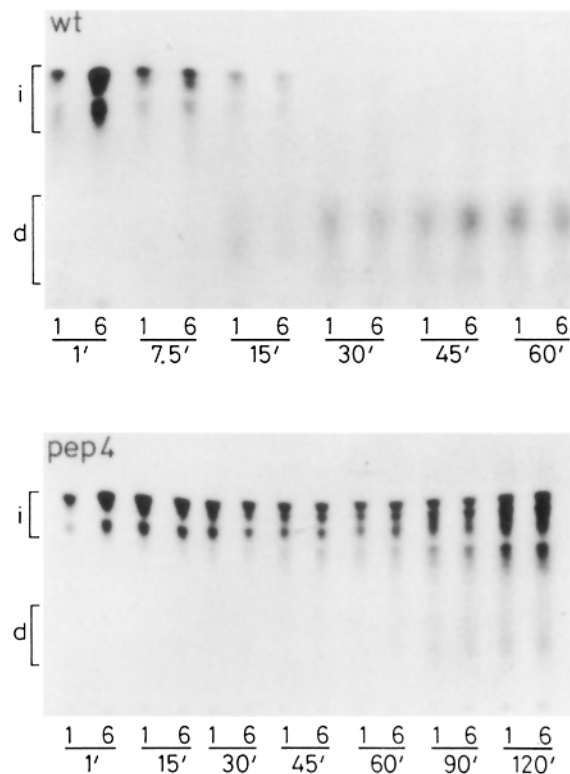
Figure 2. α-Factor degradation is PEP4 dependent. Wild-type (wt) cells (RH144-3D) and isogenic pep4 cells (RH732) were incubated with ^{35}S-α-factor (2 × 10^5 cpm/10^9 cells) for 1 h at 0°C. After removal of unbound pheromone the cells were resuspended in 30°C prewarmed, glucose-containing, potassium phosphate buffer and incubated at 30°C. At the indicated times aliquots were treated at pH 1.2 or 6 and the radioactivity was extracted from the cells as described in Materials and Methods. The extract was loaded on silica plates and resolved by TLC. The radioactivity originating from pH 1.2- or 6-treated cells is indicated by labeling the lanes 1 and 6, respectively. The positions of the intact (i) and degraded (d) α-factor are indicated. The intact α-factor consists of the authentic pheromone (R_f = 0.27) and most likely the methionine sulfoxide form of the native pheromone (R_f = 0.21) (Raths, S., Biocenter, Basel, personal communication). The fluorograms were exposed for 7 d at −70°C.
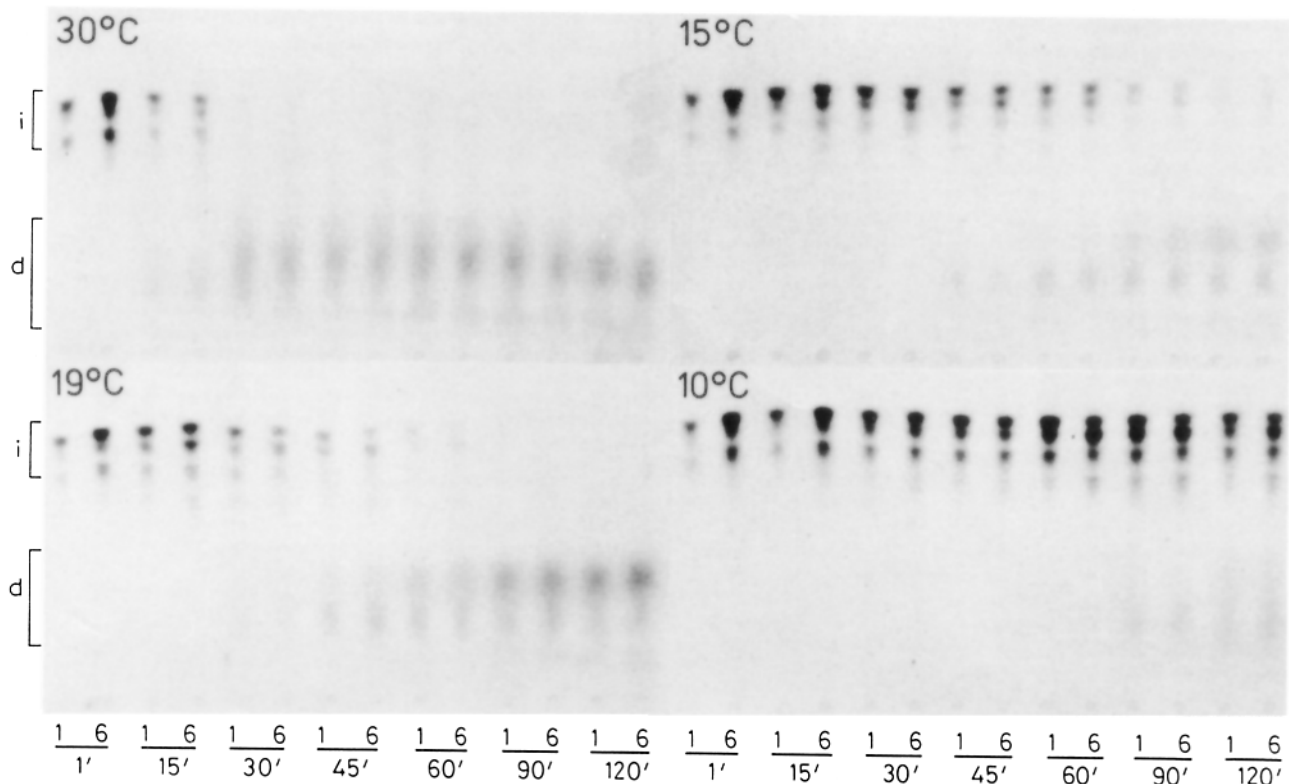
Figure 3. Effect of temperature on α-factor degradation. ^{35}S-α-factor was incubated with RH144-3D cells for 1 h on ice. The cells were harvested, resuspended in 10, 15, 19, or 30°C prewarmed, glucose-containing, potassium phosphate buffer and incubated for up to 2 h at the indicated temperature. Samples were treated at pH 1.2 (*1*) and pH 6 (*6*) at the indicated times and extracts were resolved by TLC as described in Materials and Methods. The positions of the intact (*i*) and the degraded (*d*) α-factor are shown. The fluorograms were exposed between 6 and 8 d at −70°C.

Protease Protection

The 100,000 *g* pellet (P3), that was obtained as described before with the second differential centrifugation at 10,000 *g* for 10 min, was resuspended in 90 μl 0.6 M sorbitol, 50 mM Tris, pH 8, 1 mM CaCl₂ (sorbitol buffer), and divided into three aliquots. Two aliquots were incubated with 0.5 mg/ml proteinase K either in the presence or in the absence of 0.5% Triton X-100 for 15 min at 30°C. A control aliquot was incubated without any additions. After cooling on ice, PMSF was added to 1 mM final concentration, and the samples were denatured by adding 40 μl of 3× SDS gel sample buffer and heating for 5 min at 95°C. The samples were resolved by SDS-PAGE on 17.5% polyacrylamide gels. The gels were fixed, treated with ENTEN-SIFY, dried, and subjected to fluorography at −70°C. Fluorographs were scanned as described above. Time courses of protease protection using 0.2 and 0.5 mg/ml proteinase K were also performed as described above taking samples after 15- and 30-min digestion at 30°C.

Results

Effect of Temperature on α-Factor Uptake

In mammalian cells low temperature incubations have been successfully applied to arrest delivery of endocytosed molecules to lysosomes. We therefore explored the effect of low temperature on α-factor uptake and degradation with the hope of finding conditions that would trap α-factor on its way to the vacuole. For these studies ^{35}S-α-factor was bound to a cells at 0°C for 1 h, unbound α-factor was washed away, and the cells were resuspended in prewarmed, glucose-containing, potassium-phosphate buffer at 5, 10, 15, 19, or 30°C. At various times aliquots were removed and treated at pH 6 or 1.2. The pH 6 treatment gives a measure of the total cell-associated radioactivity, whereas at pH 1.2 the surface bound radioactivity is removed and the internalized α-factor can be measured. In Fig. 1 the fraction of bound α-factor that is internalized is expressed as a function of time at the indicated temperatures. At 30°C pheromone uptake is the most rapid and complete by 30 min (Fig. 1); the time required to internalize 50% of the bound α-factor is 4 min (Table I). At 15 and 19°C the initial rate of uptake is reduced by factors of 1.8 and 2, respectively (Table I). This is only weakly impaired compared with 10°C, where pheromone uptake is reduced by a factor of 6. Only at 5°C, however, is α-factor internalization nearly blocked. During the first 5 min of the incubation at 15, 19, and 30°C, ∼20% of the surface-bound α-factor dissociates from the cells. This, however, does not affect the internalization rates calculated, because these calculations take into account both the internalized and total α-factor at every time point.

α-Factor Degradation in pep4 and Wild-type Cells as a Function of Temperature

Degradation of pheromone has been shown to be dependent on the *PEP4* gene product, proteinase A (Chvatchko, 1987; Dulić, 1989), that is responsible for activating many soluble vacuolar hydrolases (Ammerer et al., 1986; Woolford et al., 1986). Here we examine the kinetics of α-factor degradation in wild-type and *pep4* cells. In wild-type cells the intact pheromone that is internalized by the cells is completely degraded by 30 min (Fig. 2). Different degradation products appear successively between 15 and 45 min. Although *pep4*
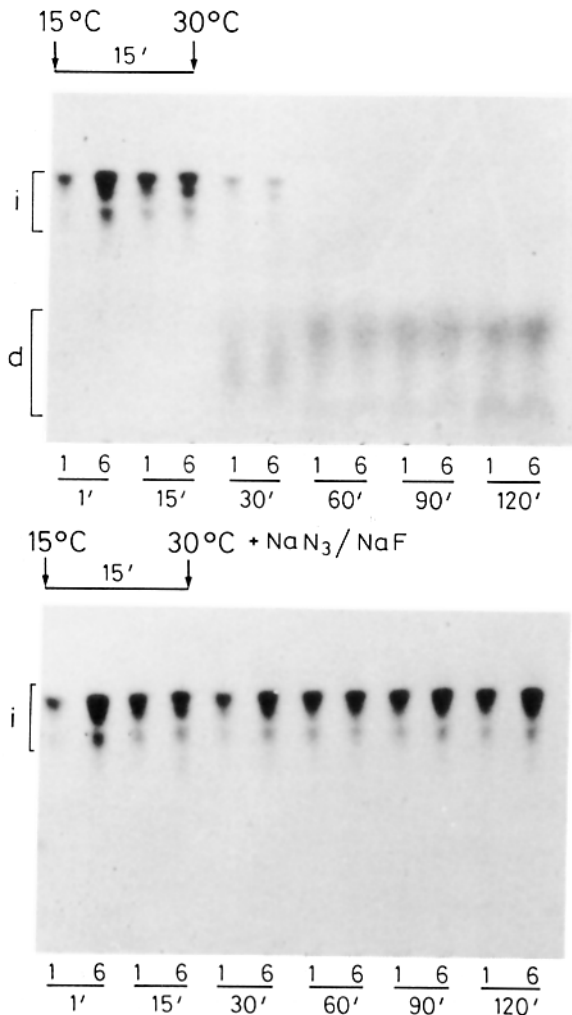
Figure 4. Metabolic inhibitors block degradation of internalized α-factor at 30°C. RH144-3D cells were incubated with ^{35}S-α-factor on ice for 1 h. After pheromone binding, cells were harvested and resuspended in 15°C prewarmed, glucose-containing, potassium phosphate buffer. After allowing uptake for 15 min at 15°C, NaN₃ and NaF were added (each 20 mM final concentration) and the cells were transferred to a 30°C waterbath. At the indicated times samples were analyzed by extracting the radioactivity from the cells and resolving the products by TLC. The position of intact (i) and degraded (d) pheromone originating from pH 1.2- (l) and pH 6- (6) treated cells is indicated. The fluorograms were exposed for 7 d at −70°C. The quantitation of this experiment is presented in Fig. 5 B.

cells internalize α-factor as efficiently as wild-type cells (data not shown), the majority of the pheromone remains intact for at least 120 min (Fig. 2, bottom). This suggests that the vacuole is the site of α-factor degradation. Upon extended incubation (90 min and more) a new species migrating just below the intact α-factor appears and a minor amount of degradation products can be seen. The accumulation of the first mentioned species, that cannot be observed in wild-type cells, might be due to an extremely low level of vacuolar hydrolase activity. Alternatively, the lack of enzymes that are responsible for rapid degradation under normal conditions could allow another nonphysiological modification to occur.

Assuming that the vacuole is the site of degradation, we used α-factor degradation as a marker for delivery of the pheromone to the vacuole. Since α-factor uptake is only moderately reduced at 19 and 15°C, it was of interest to see what effect these temperatures have on degradation. The results are presented in Fig. 3. Lowering the temperature reduces the rate of α-factor degradation. The time necessary to degrade 50% of the internalized, intact α-factor is shown in Table I. At 19°C the degradation and uptake rates are reduced to a similar extent. However, at 15 and 10°C degradation is more severely affected than uptake. In both cases the effect of temperature on degradation is two fold greater than on uptake (Table I). This finding can be explained in two ways. First, a step in the α-factor degradation pathway subsequent to uptake may be more temperature sensitive than uptake. This step could be a vesicular transport step from a prevacuolar compartment to the vacuole. Alternatively, one could argue that the primary reason for the delay in α-factor degradation is not a transport problem, but simply due to reduced vacuolar hydrolase activity at low temperature.

Degradation of α-Factor Can Be Blocked by Metabolic Inhibitors

To demonstrate that the reduced rate of α-factor degradation at 15°C is not solely due to reduced vacuolar hydrolase activity at this low temperature, we tested whether α-factor that has been internalized at 15°C can be degraded in the presence of metabolic inhibitors at 30°C. If the pheromone is already transported to the vacuole at 15°C, it should be degraded at 30°C, even in the presence of NaN₃ and NaF, since ATP is not required for the enzymatic activity of vacuolar hydrolases. After allowing uptake for 15 min at 15°C, the metabolic poisons were each added to a final concentration of 20 mM and the cells were transferred to a 30°C waterbath. Upon shift to 30°C, in the absence of NaN₃ and NaF, α-factor is degraded, demonstrating that the effect of temperature is reversible (Fig. 4). However, in the presence of metabolic inhibitors degradation is completely inhibited (Fig. 4). The same degree of inhibition can be achieved using 5 mM each NaN₃ and NaF (data not shown). The argument could be made that a raised vacuolar pH, due to the presence of the metabolic inhibitors, might be the primary reason for the block in α-factor degradation seen in the NaN₃- and NaF-treated cells. To exclude this possibility we investigated α-factor degradation in cells that had been treated with the specific and potent inhibitor of the vacuolar H⁺-ATPase, bafilomycin A₁ (Bowman et al., 1988). To confirm that the drug actually raises the vacuolar pH under the applied conditions, cells were stained with quinacrine, a fluorescent dye that accumulates in acidified organelles (Weisman et al., 1987). 10, 45, and 105 min after the addition of bafilomycin A₁ the cells did not show any staining with quinacrine while control cells did (data not shown), demonstrating that the vacuolar pH was raised by the drug. This is in agreement with the results described by Banta et al. (1988), and also confirms that the inhibitor is active over the whole time course of the experiment. Addition of DMSO alone has almost no inhibitory effect on α-factor degradation, and treatment with bafilomycin A₁ delays but does not block degradation (Fig. 5 A). Quantitation of the internalized, intact α-factor (Fig. 5 B, open and solid circles) reveals that bafilomycin A₁-treated cells exhibit an immediate delay in degradation upon addition of the
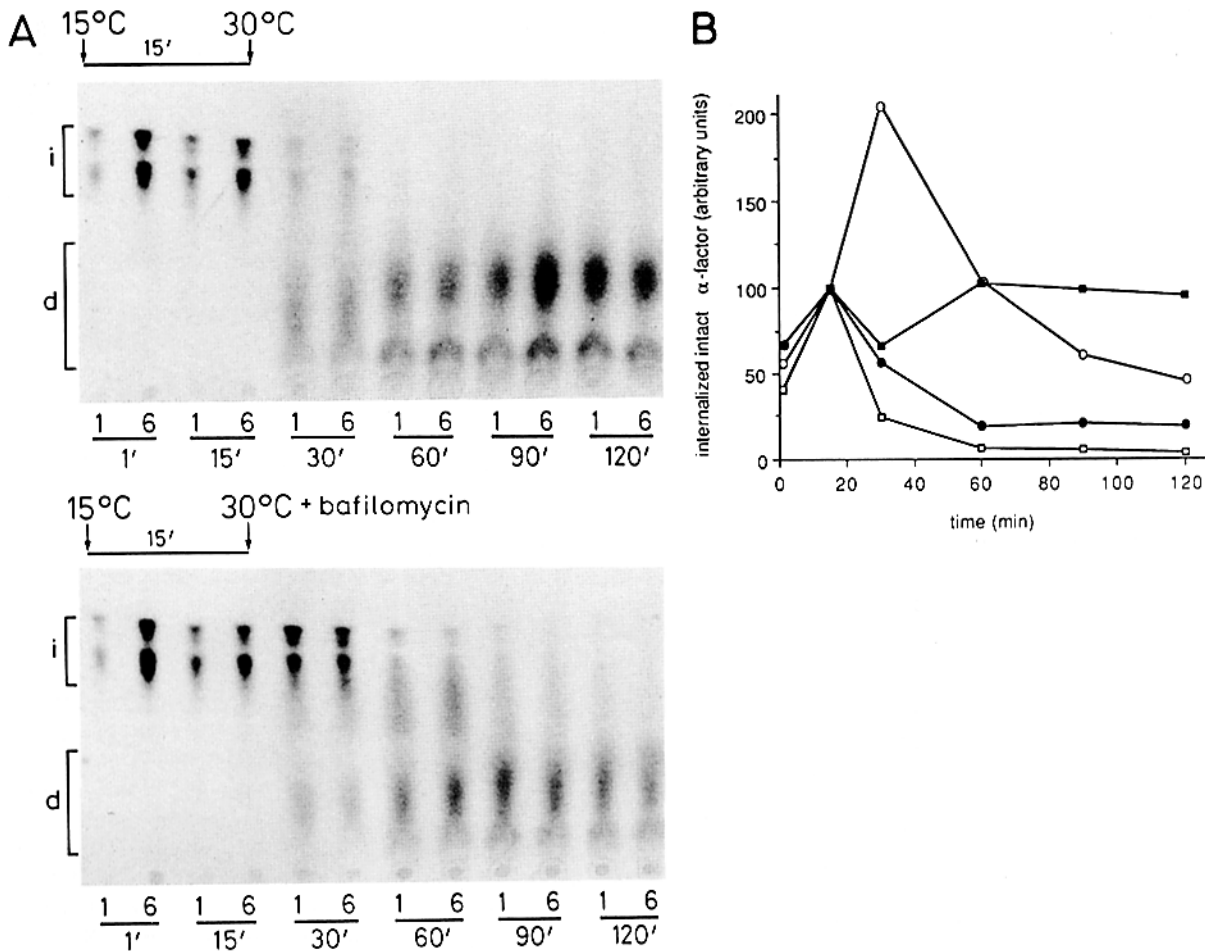
Figure 5. Bafilomycin A₁ does not block α-factor degradation. (A) The experiment was done as described in Fig. 4. Instead of NaN₃ and NaF, bafilomycin A1 was added to 10-μM final concentration where indicated. Control cells (*top*) were treated with DMSO alone. (B) The amount of intact α-factor (*i*) that is extracted from the pH 1.2 treated cell samples was quantified by densitometric scanning of the fluorographs shown in A and Fig. 4 (see Materials and Methods) and is expressed as a function of time. The values obtained from the experiment described in A are symbolized by the open and solid circles. The values obtained from the experiment described in Fig. 4 are symbolized by the open and solid squares. (o) + bafilomycin; (●) – bafilomycin; (■) + NaN₃, + NaF; (□) – NaN₃, – NaF.

drug. This is noticed from the superaccumulation of internalized, intact α-factor. After ∼30 min the intact α-factor starts to disappear with perhaps slightly reduced kinetics in comparison to control cells. Fig. 5 B also presents the quantitation of intact α-factor in the NaN₃- and NaF-treated and untreated cells, shown in Fig. 4. The respective values are symbolized by the open and solid squares (Fig. 5 B), and clearly demonstrate that the metabolic inhibitors exert a qualitatively and quantitatively different effect on α-factor degradation than bafilomycin A₁. These results support the idea that the lack of pheromone degradation, observed in the presence of metabolic poisons, is not solely due to vacuolar pH but is more likely due to a block in transport from an endocytic, prevacuolar compartment to the vacuole.

To determine the kinetics of delivery of α-factor to the vacuole at 15°C, cells were incubated with prebound α-factor for various periods at 15°C before they were shifted to 30°C for an additional incubation in the presence of metabolic inhibitors. Extracts of cells were analyzed after a total incubation time of 120 min. This experiment shows that the cells can be left for at least 30 min at 15°C without detecting any

α-factor degradation (Fig. 6). Only at or after 45 min at 15°C can one detect the appearance of degradation products. After any time period at 15°C the additional incubation at 30°C in the presence of NaN₃ and NaF results in very little further degradation of α-factor (compare with Fig. 3). This suggests that α-factor degradation is not limiting at 15°C in the absence of metabolic inhibitors. The α-factor must be very rapidly degraded upon delivery to the vacuole. Moreover, these experiments suggest that from 15 to 30 min at 15°C, when most of the α-factor is internalized, there is no delivery of pheromone to the vacuole.

Inhibition of α-Factor Degradation in NaN₃- and NaF-treated Cells Is Reversible

Since the preceding results imply that a combination of low temperature and NaN₃ and NaF treatment can result in a block in transport of α-factor to the vacuole, we asked whether this block is reversible. α-Factor was internalized at 15°C for 15 min and the cells were transferred to 30°C in the presence of NaN₃ and NaF. After a 15-min incubation to impose the block, the cells were harvested, resuspended
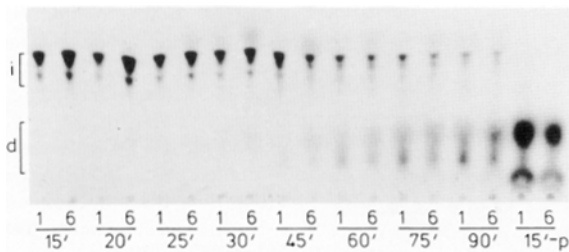
Figure 6. Addition of metabolic inhibitors after various times of α-factor internalization. RH144-3D cells were incubated with ^{35}S-α-factor for 1 h on ice. After pheromone binding the cells were harvested and resuspended in 15°C prewarmed, glucose-containing, potassium phosphate buffer at time zero. At the indicated times aliquots of cells were shifted to 30°C and NaN$_3$ and NaF were added (a control sample was incubated in the absence of metabolic poisons [p]). After a total incubation time of 120 min (including uptake at 15°C and further incubation at 30°C) the state of the α-factor was analyzed by extracting the radioactivity and resolving the products by TLC. The fluorograms were exposed for 7 d at −70°C. The position of intact (i) and degraded (d) α-factor originating from pH 1.2- (1) and pH 6-(6) treated cells is indicated.

in inhibitor-free medium, and further incubated at 30°C. As shown in Fig. 7, 30 min after removal of NaN$_3$ and NaF the α-factor is almost completely degraded. Only a minor fraction of the intact species is not degraded. This finding suggests that the majority of the undegraded α-factor, accumulating in the NaN$_3$- and NaF-treated cells, is trapped on the transport pathway to the vacuole (see below) and can be subsequently chased through it upon removal of the metabolic inhibitors.
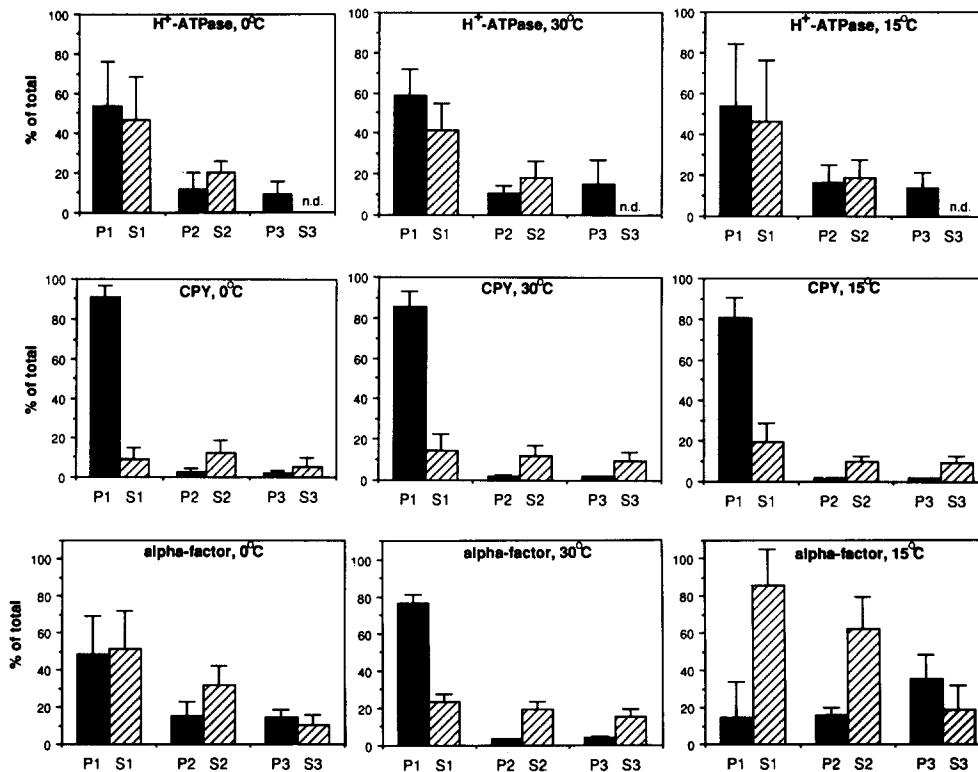
Sedimentation Behavior of α-Factor After Uptake at Different Temperatures

To confirm the idea that pheromone transport from the plasma membrane to the vacuole passes through a prevacuolar compartment, we analyzed the sedimentation behavior of α-factor after different uptake regimes and compared it with a vacuolar, a plasma membrane, and a cytosolic protein marker. The pheromone was either bound to the plasma membrane at 0°C or was allowed to be internalized at 30°C for 30 min or at 15°C for 20 min. We hypothesized that α-factor internalized at 30°C for 30 min would cofractionate with a vacuolar marker, as under these conditions it is completely degraded, whereas when uptake is performed at 15°C it would not fractionate with the vacuole. For these experiments *pep4* cells were used to avoid nonspecific protein and α-factor degradation during the fractionation. After pheromone uptake, the cells were converted into spheroplasts at 30°C in the presence of 5 mM of both NaN$_3$ and NaF, to inhibit further pheromone transport. Cells were broken using polybase-induced lysis with DEAE-dextran as described by Dürr et al. (1975). This gentle lysis procedure is especially advantageous to analyze fragile organelles. The lysate was subjected to three successive centrifugation steps. First, it was centrifuged at 3,500 g for 5 min, giving a low speed pellet P1 and a low speed supernatant S1. This supernatant was centrifuged at 10,000 g for 10 min, resulting in P2 and S2. The S2 was finally subjected to 100,000 g for 1 h, giving rise to P3 and S3. The distribution of α-factor and three protein

markers in the six fractions was quantified by counting the radioactivity of ^{35}S-α-factor and by Western blotting, respectively. Plasma membrane was detected using an antiserum against the plasma membrane H$^+$-ATPase, and the distribution of the vacuole was followed by an antiserum against CPY. To correct for the varying extent of cell lysis we additionally quantified the distribution of the cytoplasmic marker hexokinase (as described in Materials and Methods). The distribution of the described markers in the six fractions is presented in Fig. 8. The plasma membrane H$^+$-ATPase is found most prevalently, and under all conditions (0, 15, and 30°C), in P1 (50–60%). The remaining amount is found in approximately equal quantities in P2 and P3. The soluble vacuolar marker, CPY, sediments under all conditions predominately at 3,500 g. This demonstrates that most of the vacuoles remain intact under the described lysis conditions. Most of the remaining CPY (∼15%) is found in the high speed supernatant S3, whereas in P2 and P3 only very little can be detected. To rule out that the incubation of cells in the presence of metabolic poisons might influence or change the distribution of these two markers, spheroplasts were prepared in the absence of NaN$_3$ and NaF, lysed, and fractionated as described before. As expected, we obtained results similar to those described above (data not shown), implying that treatment with NaN$_3$ and NaF does not alter the sedimentation behavior of CPY and the plasma membrane H$^+$-ATPase. Whereas the distribution of the vacuolar and plasma membrane markers do not change with the various conditions, the distribution of ^{35}S-α-factor does. After incubation at 0°C the radioactivity cofractionates with the plasma membrane H$^+$-ATPase. When the pheromone is internalized at 30°C, its sedimentation behavior is similar to that of CPY, providing further evidence that α-factor is transported to the vacuole, where degradation takes place. Interestingly, when α-factor uptake is done at 15°C, the pheromone does not
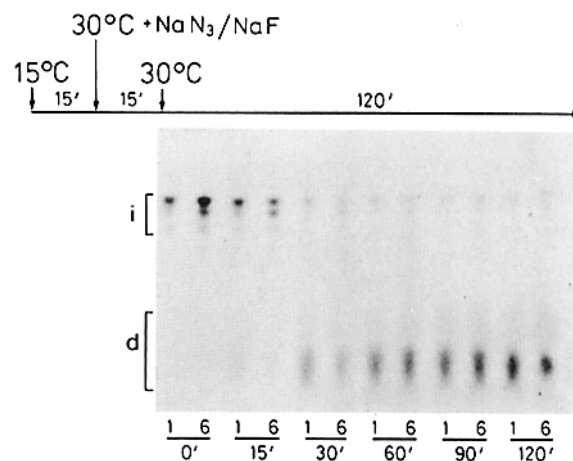
Figure 7. The block in α-factor degradation due to NaN$_3$ and NaF is reversible. Binding and uptake of ^{35}S-α-factor in RH144-3D cells was done as described in the legend to Fig. 4. 15 min after the addition of 20 mM of both NaN$_3$ and NaF and the shift to 30°C, the cells were harvested and resuspended in the same volume of inhibitor-free medium and further incubated at 30°C. The radioactivity was extracted at the indicated time points. The position of intact (i) and degraded (d) pheromone is marked. The fluorogram was exposed for 8 d at −70°C.

Figure 8. Sedimentation behavior of CPY, the plasma membrane H^+-ATPase, and α-factor after pheromone binding at 0°C and uptake at 15 and 30°C. RH732 cells were incubated with ^{35}S-α-factor for 1 h at 0°C in complete medium. The cells were either left at 0°C for 30 min, or were warmed up to 15°C for 20 min, or to 30°C for 30 min. The cells were converted into spheroplasts in the presence of 5 mM each NaN_3 and NaF as described in Materials and Methods. The spheroplasts were centrifuged through a 0.6-M sucrose cushion, resuspended in sorbitol medium, and lysed as described in Materials and Methods. The lysate was centrifuged at 3,500 g for 5 min, resulting in P1 and S1. S1 was centrifuged at 10,000 g for 10 min, giving rise to P2 and S2; S2 was finally subjected to 100,000 g for 60 min, resulting in P3 and S3. Aliquots from each of the six fractions were counted for ^{35}S-α-factor and proteins were resolved by SDS-PAGE. Vacuolar CPY, the plasma membrane H^+-ATPase, and cytoplasmic hexokinase were detected by Western blot analysis and quantified by densitometric scanning (see Materials and Methods). The individual values for each marker were corrected for cell lysis as described in Materials and Methods. The error bars represent the standard deviation of the mean of at least five experiments at every condition. *n.d.*, not detectable.

fractionate like CPY nor like the plasma membrane H^+-ATPase. The largest fraction sediments at 100,000 g (35% in P3) and the remaining amount is found almost equally in P1, P2, and S3. This result clearly demonstrates that the α-factor taken up at 15°C is not associated with the vacuole nor with the plasma membrane, but may be trapped within an intermediate compartment(s) (see below). The fact that this compartment sediments at 100,000 g is consistent with it being a small organelle.

α-Factor That Is Sedimentable at 100,000 g Is Membrane Enclosed

To determine whether the pheromone found in the 100,000 g pellet (P3) resides within a membrane-sealed compartment, we investigated its sensitivity to protease digestion. Aliquots from the P3 fraction were either left untreated or incubated with proteinase K in the presence or absence of Triton X-100. The reaction products were separated on 17.5% SDS–polyacrylamide gels that were analyzed by fluorography and densitometry. By using 0.5 mg/ml proteinase K in the presence of Triton X-100 (Fig. 9, lane 2), all of the α-factor is degraded after a 15-min incubation, while 66% of the pheromone is protected from protease digestion in the absence of detergent (lane 3). This provides evidence that α-factor is localized within a membrane-sealed vesicle with the orientation expected for a transport vesicle. The fact that only 66% of the α-factor is protected in comparison to the protease untreated sample (Fig. 9, lane 1) may be explained by some damage of vesicles caused by resuspension of the P3. With a smaller

amount of proteinase K (0.2 mg/ml) in the presence of Triton X-100, 70% of the α-factor is degraded after 15 min and almost all is degraded after 30-min incubation at 30°C (data not shown). This shows that proteolysis is limited by the amount of proteinase K and not due to unspecific protease activity that could be released by Triton X-100.

Another line of evidence confirming the idea that α-factor is contained within a vesicular compartment is supplied by floatation analysis of the P3 fraction on a Nycodenz gradient. In this experiment the S2 fraction was obtained after centrifugation of the lysate at 3,500 and 7,500 g, each for 5 min, in order to optimize the recovery of α-factor in the high speed pellet (P3). The density gradient was subjected to centrifugation at 100,000 g for 14–16 h to allow the vesicles to reach their equilibrium density, then was fractionated, and the amount of ^{35}S-α-factor, H^+-ATPase, and protein in each fraction quantified. The results of one typical experiment are presented in Fig. 10. The α-factor is present in two peaks at densities of $\sim$1.12 and 1.14 g/ml, respectively, both of which are clearly resolved from the bulk protein. Moreover, the distribution of α-factor and the plasma membrane H^+-ATPase, peaking predominately at a density of 1.16 g/ml, are clearly distinct, supporting again the idea that the α-factor is not associated with the plasma membrane.

Discussion

Reduction of temperature and metabolic inhibitors have been used extensively to arrest membrane transport at defined

1	2	3

Proteinase K   — + +

Triton X-100   — + —
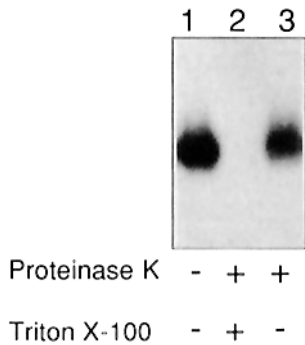
Figure 9. ³⁵S-α-factor that is associated with P3 after uptake at 15°C is protease protected. ³⁵S-α-factor uptake at 15°C and preparation of subcellular fractions were carried out as described in the legend to Fig. 8. The high-speed pellet (P3) was resuspended in 0.6 M sorbitol buffer and incubated with or without 0.5 mg/ml proteinase K in the presence or absence of 0.5% Triton X-100, as indicated, at 30°C for 15 min. The reaction was stopped by chilling the samples on ice and addition of 1 mM PMSF. The samples were resolved by SDS-PAGE on a 17.5% gel. The amount of ³⁵S-α-factor in each lane was quantified by densitometric scanning of the fluorogram. The fluorogram was exposed for 2 d at −70°C.

stages in the endocytic and exocytic pathways in mammalian cells (reviewed by Mellman et al., 1986; Griffiths and Simons, 1986; Pfeffer and Rothman, 1987). In this study we have examined the effect of low temperature and metabolic poisons on transport of the yeast pheromone α-factor to the vacuole. There are several lines of evidence that are consistent with the notion that α-factor degradation takes place in the vacuole. First, degradation is dependent on the *PEP4* gene product, proteinase A. This protease is essential for vacuolar zymogen activation, so that cells carrying mutations in the *PEP4* gene have a major defect in vacuolar hydrolytic activity (Ammerer et al., 1986; Wollford et al., 1986). Although *pep4* cells internalize α-factor as efficiently as wild-type cells, the majority of the pheromone remains intact. In agreement with this, a mutant that lacks a functional vacuole (*end1*) also does not degrade α-factor, although the pheromone is internalized normally (Dulić and Riezman, 1989). Second, analysis of the sedimentation behavior of α-factor from lysates of cells that have internalized the pheromone for 30 min at 30°C show a nearly perfect codistribution of α-factor and the vacuolar marker CPY. Even though the P1 fraction, where the majority of the α-factor and CPY are found, is not a pure fraction, we show here that at least plasma membrane fractionates differently. Another organelle that should be found predominately in this fraction is the nucleus. However, we think it is very unlikely that the pheromone is associated with the nucleus, as the enzymes that are activated by the *PEP4* gene product are not found there (Wiemken et al., 1979; van Tuinen and Riezman, 1987). Third, an elevated pH of the vacuolar system apparently slows down pheromone degradation. This is similar to what has been observed in mammalian cells, using lysosomotropic agents and carboxylic ionophores (reviewed by Mellman et al., 1986).

Using α-factor degradation as a marker for transport to the vacuole, we have established conditions that allow α-factor uptake, but result in a significantly reduced rate of delivery to the vacuole. At 19°C the rates of uptake and degradation are similarly reduced in comparison to 30°C. However, at 15 and 10°C degradation is proportionally more delayed than uptake (Table I). Our data favor the hypothesis that this delay in degradation is due to a kinetic block of transport to the vacuole, rather than to an inhibition of vacuolar hydrolytic activity. First, α-factor that is taken up at 15°C for 20 min

does not cofractionate with the vacuolar marker CPY, whereas after internalization at 30°C for 30 min it does. Second, when cells that have internalized α-factor for 15 min at 15°C are shifted to 30°C, the time required for degradation products to appear is similar to when the uptake is carried out exclusively at 30°C. If α-factor already resided in the vacuole after uptake at 15°C, one would not expect to find a 15-min lag in degradation. As α-factor degradation seems not to be limiting in our assay, these data suggest that the rate limiting step in the appearance of degradation products, even at 30°C, is the transport of α-factor to the vacuole. This is consistent with results obtained with mammalian cells, which show that fusion between endocytic vesicles and lysosomes in the perfused rat liver is the slowest step that occurs during the catabolism of asialoglycoprotein. This step is blocked at temperatures below 20°C (Dunn et al., 1980). A similar result was obtained by Marsh et al. (1983), who characterized the entry pathway of Semliki Forest virus in
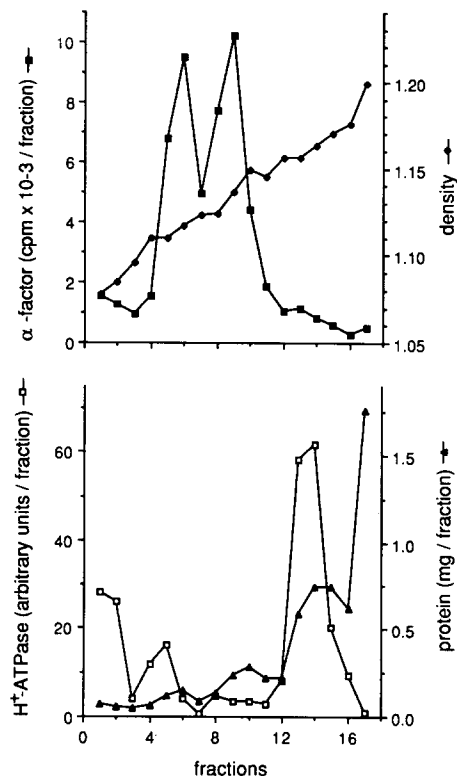
Figure 10. Floatation analysis of P3 on a density gradient. The P3 fraction containing the ³⁵S-α-factor after uptake at 15°C was used for the floatation analysis and was prepared in the same way as described in Fig. 8 with the exception that the second differential centrifugation step was at 7,500 g for 5 min. The P3 fraction was resuspended in 35% Nycodenz, 1 ml was placed into a centrifugation tube and overlaid with each 2.5 ml of four different Nycodenz dilutions in sorbitol medium as described in Materials and Methods. The gradient was centrifuged at 100,000 g for 14–16 h at 4°C and was collected from the top. Aliquots from each of the 17 fractions were analyzed for ³⁵S-α-factor (■), H⁺-ATPase (□), protein concentration (▲), and density (◇) as described in Materials and Methods. Only 40% of protein applied to the gradient was recovered, most likely due to the fact that a major part of the pellet formed during centrifugation was not collected. However, >95% of the radioactivity was recovered.

baby hamster kidney cells. In their system, transport of viral proteins from endosomes to lysosomes is also blocked at 20°C. The finding that endocytic traffic in yeast seems to be efficiently slowed down only at 15°C, whereas in mammalian cells it is reduced at 20°C, is not surprising, because yeast cells normally grow at lower temperatures than mammalian cells.

In conjunction with low temperature incubations, we made use of the metabolic inhibitors NaN₃ and NaF to block transport from a prevacuolar compartment(s) to the vacuole. Both treatments are necessary to completely block the transport from this compartment, since low temperature installs only a kinetic block. The effect of the metabolic inhibitors on α-factor transport is consistent with in vitro experiments that reconstitute parts of mammalian endocytic (Davey et al., 1985; Gruenberg and Howell, 1986; Smythe et al., 1989) and exocytic transport (Balch et al., 1984). These studies showed that budding and fusion of vesicles are dependent on ATP. In our experiments we found that 5 mM NaN₃ and NaF were sufficient to block degradation in vivo and transport of α-factor to the vacuole, even at 30°C. It is unlikely that the metabolic poisons inhibit degradation by increasing the vacuolar pH and thereby inhibiting the vacuolar hydrolytic enzyme activity. Brief treatment of cells with bafilomycin A₁ results in an increase of vacuolar pH, which can be monitored by the lack of vacuolar labeling with quinacrine. However, bafilomycin A₁ does not block α-factor degradation in contrast to treatment with NaN₃ and NaF. This permits us to conclude that the lack of α-factor degradation, seen in the presence of metabolic inhibitors, is not solely due to an elevated vacuolar pH, but to a block in delivery to the vacuole. The finding that bafilomycin A₁ changed the kinetics of α-factor degradation in comparison to control cells cannot be explained at the moment, but is currently being investigated in more detail.

Direct evidence that α-factor transport from the plasma membrane to the vacuole involves at least one intermediate compartment comes from analysis of the sedimentation behavior of the pheromone internalized under various conditions. Pheromone that was incubated with cells under conditions where we predicted an association with either plasma membrane or vacuole does in fact cofractionate with the plasma membrane H⁺-ATPase and CPY, respectively. α-Factor that is internalized at 15°C under conditions where no degradation products can be detected, has a sedimentation behavior that is strikingly different from that of the vacuole or plasma membrane markers. The largest proportion of the α-factor is found in the 100,000-g pellet, but some of it is also associated with the 3,500-g pellet, the 10,000-g pellet, and the 100,000-g supernatant. At the moment we can only speculate about the origin of the pheromone found in these fractions. α-Factor that is pelletable at 3,500 g could be associated with the plasma membrane. After a 20-min incubation at 15°C ∿20% of pheromone is still sensitive to a pH 1.2 wash (Fig. 1) and ∿50–60% of the plasma membrane pellets at 3,500 g. By using the same type of calculation only a minor amount of pheromone found in the 10,000- and 100,000-g pellets could be derived from the plasma membrane. In addition, only very small quantities of pheromone present in these fractions could originate from the vacuole. Therefore, we conclude that most of the radioactivity sedimenting into P2 and P3 originates from α-factor residing within the intermediate compartment(s).

Floatation analysis of the 100,000-g fraction, obtained after α-factor uptake at 15°C, reveals that the pheromone is present in two peaks. This demonstrates conclusively that the pheromone is associated with at least one membranous compartment that is distinct from the plasma membrane. The two α-factor-containing peaks could represent two different compartments involved in transport to the vacuole. However, the possibility that they represent the same compartment in an intact and damaged form cannot be excluded at this point.

The existence of an intermediate, endocytic compartment in S. cerevisiae has previously been claimed by Makarow and Nevalainen (1987) using commercially available FITC-dextran. Subsequently, Preston et al. (1987) demonstrated that intracellular labeling under these conditions is due to low molecular weight impurities in the commercially available FITC-dextran. Therefore, the labeling seen by Makarow and Nevalainen cannot be considered to be endocytic in origin. Makarow (1985a) has also proposed that Semliki Forest virus and vesicular stomatitis virus, well-established endocytic markers in mammalian cells (Helenius et al., 1980), are taken up by endocytosis into yeast spheroplasts. After internalization and cell lysis three density gradient fractions were claimed to contain the viral proteins, one of which coincided with vacuolar markers, one with plasma membrane markers, and one banded between the vacuolar and the plasma membrane markers. Makarow considered either or both of the latter fractions to represent intermediary compartments operating in transport to the vacuole. However, the evidence that virus is delivered to the vacuole is not convincing. Approximately the same proportion of viral radioactivity and plasma membrane marker cofractionate with the vacuolar marker. This calls into question this interpretation and perhaps the use of these viruses as endocytic markers in yeast.

If the intermediate compartment(s) that we propose is endocytic in origin then one would expect the α-factor to reside within this organelle(s). Evidence in support of this is provided by the finding that the pheromone present in P3 after uptake at 15°C is protease protected. High concentrations of protease and incubation at 30°C for 15 min are necessary to degrade all the α-factor in the presence of Triton X-100 whereas in vitro the pure pheromone is more sensitive to protease (Singer, B., unpublished observation). We speculate that this might reflect a tight association of α-factor with some other protein(s). If the pheromone would be freely diffusable within the vesicle, such high concentrations and temperatures would presumably not be necessary for degradation. Since α-factor is most likely taken up by receptor-mediated endocytosis, the α-factor receptor is certainly a good candidate for a protein to which the pheromone might be bound. α-Factor, because it is rather small, could be buried by parts of the receptor, so that only after protease digestion of the receptor would α-factor become accessible to the protease.

It still remains to be demonstrated that the α-factor receptor is internalized. The first hint, that the receptor is taken up by ligand-induced endocytosis, comes from studies by Jenness and Spatrick (1986) demonstrating that α-factor binding activity disappears from the surface concomitant with pheromone uptake. Analysis of receptor truncation mutants has revealed that COOH-terminal sequences of the

receptor are not necessary for pheromone binding, yet function to regulate receptor internalization and adaptation to pheromone by phosphorylation (Konopka et al., 1988; Reneke et al., 1988). Recently, Dietzel and Kurjan (1987) and Miyajima et al. (1987) provided the first evidence for a receptor-coupled G-protein in yeast, that is involved in mating. The G-protein subunits show sequence homology to mammalian receptor-coupled G-proteins. In addition to this similarity, the α-factor receptor and some G-protein-coupled receptors like rhodopsin and the β-adrenergic receptor share striking structural homology, in that they all contain seven transmembrane domains, and NH_2-terminal exoplasmic, and a COOH-terminal cytoplasmic domain. Evidence has accumulated that suggests that the β-adrenergic receptor is internalized (called sequestration) (reviewed by Sibley et al., 1987). However, the mechanism of internalization and subsequent targeting of polytopic signal transduction receptors has not been clarified yet in any system. One clue from yeast may come from work on clathrin-deficient mutants. They are still able to carry out α-factor uptake, although it is reduced by 50–65% (Payne et al., 1988). Besides the inherent difficulty to interpret this partial effect, there is no reason to believe at the present time that clathrin is strictly required for receptor-mediated endocytosis, nor is there convincing evidence that polytopic signal transduction receptors are internalized via clathrin-coated pits. Since we are now in a position to isolate and characterize the intermediate compartment(s) that is involved in transport of α-factor to the vacuole, we hope to learn more soon about the molecular requirements of the endocytic transport pathway in yeast.

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