Cis- and Trans-acting Functions Required for Endocytosis of the Yeast Pheromone Receptors

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Abstract. The Saccharomyces cerevisiae a-factor receptor (STE3) is subject to two modes of endocytosis: a constitutive process that occurs in the absence of ligand and a regulated process that is triggered by binding of ligand. Both processes result in delivery of the receptor to the vacuole for degradation. Receptor mutants deleted for part of the COOH-terminal cytoplasmic domain are disabled for constitutive, but not ligand-dependent internalization. Trans-acting mutants that impair constitutive endocytosis have been isolated. One of these, renl-1, is blocked at a late step in the endocytic pathway, as receptor accumulates in a pre-vacuolar endosome-like compartment. REN1 is identical to VPS2, a gene required for delivery of newly synthesized vacuolar enzymes to the vacuole. Based on this identity, we suggest a model in which the transport pathways to the vacuole—the endocytic pathway and the vacuolar biogenesis pathway—merge at an intermediate endocytic compartment. As receptor also accumulates at the surface of renl cells, receptor may recycle from the putative endosome to the surface, or REN1 may also be required to carry out an early step in endocytosis.

Endocytosis of cell surface receptors plays a vital role in cell physiology. Endocytosis of some receptors, for example, the transferrin or LDL receptors, is the first step in the delivery of essential nutrients to the cell. Endocytosis of other receptors, particularly hormone receptors, serves to control receptor abundance and therefore the cell's sensitivity to hormone. Most receptor-mediated endocytosis is thought to occur via clustering of receptors at clathrin-coated pits, which pinch off from the surface giving coated vesicles. Once internalized, receptor and ligand pass through early and late endosomal compartments. From these compartments the endocytic pathway branches, allowing either recycling back to the cell surface or delivery to the lysosome for hydrolysis (for reviews see Goldstein et al., 1985; Schlessinger, 1988; Gruenberg and Howell, 1989; Kornfeld and Melman, 1989; Griffiths and Gruenberg, 1991; Pfeffer, 1992).

Many features of endocytosis, including the requirement for initial association with clathrin pits, vary among receptor types. Some receptors, for example the low-density lipoprotein (LDL) receptor, associate spontaneously with the pits and are internalized at a constant rate irrespective of their liganded state (Anderson et al., 1978). Other receptors, for example the EGF receptor, associate with clathrin pits only when bound by ligand, and thus it is the ligand binding event that triggers endocytosis (Schlessinger et al., 1978; Maxfield et al., 1978). Receptor types also have different destinies once internalized. LDL and its receptor dissociate in the early endosome. The receptor is then recycled to the surface while the ligand is delivered to the lysosome for hydrolysis (Basu et al., 1981). On the other hand, both EGF and its receptor are delivered to and degraded in the lysosome (Carpenter and Cohen, 1976).

Although there is a sophisticated picture of the initial events of endocytosis and the subsequent membrane traffic, the mechanisms that underlie and regulate the processes are poorly understood. In particular, aside from clathrin and clathrin-associated proteins termed adaptins (Pearse and Robinson, 1984), which are involved in coated pit formation, and rab proteins, a class of GTP-binding proteins some of which have been found associated with early and late endosomes (Chavrier et al., 1990; van der Slijs et al., 1991, 1992; Bucci et al., 1992), the molecules that catalyze and control each step of the endocytic pathway are unknown. With the expectation that a genetic analysis would contribute to the identification of such molecules, we have investigated the internalization of the pheromone receptors of the yeast Saccharomyces cerevisiae and have begun to isolate mutants defective for internalization. Pheromone receptors enable cell-cell communication as a prelude to mating of the two haploid cell types, a and alpha (Herskowitz, 1989). Each cell type secretes a unique peptide pheromone—a-factor by a cells and alpha-factor by alpha cells—and expresses at its surface a receptor for the pheromone secreted by the other cell type. Binding of pheromone to its cognate receptor activates an in-

1. Abbreviations used in this paper: ALP, alkaline phosphatase; CPY, carboxypeptidase; DB, digestion buffer; LDL, low-density lipoprotein; SB, sample buffer; vps, vacuolar protein sorting.
tracellular signal transduction pathway that leads to the physiological alterations that permit mating.

The pheromone receptors are members of a large family of G protein-coupled receptors initially defined by the β-adrenergic receptor and the rhodopsins (Dohlman et al., 1991). Although receptors of this family often show little sequence identity, they do share structural similarities. In particular, they are characterized by an NH2-terminal domain with seven hydrophobic, presumably membrane-spanning segments followed by a hydrophilic COOH-terminal domain oriented toward the cytoplasm. In cases where it has been examined, deletion of the bulk of the COOH-terminal domain does not interfere with ligand binding or G protein coupling, indicating that the seven transmembrane segment domain suffices for these functions. Deletion of the receptor COOH-terminal domain results in a heightened and prolonged response to ligand, implying that this domain functions to attenuate receptor activity (Konopka et al., 1988; Reneke et al., 1988; Dohlman et al., 1991; C. Boone, N. Davis, and G. Sprague, manuscript submitted for publication).

Endocytosis of the seven transmembrane segment receptor family has not been extensively studied. Treatment of animal cells with β-adrenergic agonists results in a rapid loss of surface binding sites, implying that the receptor has been internalized (Benovic et al., 1988). Whether these receptors associate with clathrin pits has not been investigated. Studies with yeast have suggested that the α-factor receptor is also likely subject to endocytosis. First, treatment with α-factor results in the loss of surface binding sites (Jenness and Spatrick, 1986). Second, the α-factor ligand is internalized and degraded by a pathway that involves an intracellular vesicular compartment, perhaps an endosome (Jenness and Spatrick, 1986; Chvatchko et al., 1986; Singer and Riezman, 1990). Because degradation of α-factor requires vacuolar protease activity, it is presumed that the ligand is delivered to the vacuole, an organelle equivalent to the lysosome. Third, disruption of the yeast clathrin heavy chain gene (Payne and Schekman, 1985; Lemmon and Jones, 1987) reduces the rate of α-factor uptake (Payne et al., 1988). Recently several mutants that block either the uptake or degradation of α-factor have been isolated (Wichmann et al., 1992; Raths et al., 1993). However, for neither wild-type cells nor these endocytosis mutants has the fate of the receptor itself been investigated.

In this paper we demonstrate endocytosis of the pheromone receptors by examining the receptor protein directly. We focus on the α-factor receptor and measure its stability and location in the presence and absence of pheromone. We conclude that this receptor is subject to two apparently discrete modes of endocytosis, both of which deliver the protein to the vacuole for degradation. One mode is constitutive, occurring in the absence of α-factor ligand, whereas the second mode is triggered by ligand. Receptor mutants deleted for part of the COOH-terminal domain are defective for constitutive endocytosis but normal for ligand-stimulated endocytosis. We also report the isolation of trans-acting mutations that block receptor endocytosis or subsequent steps in the endocytic pathway (ren mutations). Our characterization suggests that the wild-type REN1 product acts relatively late in the endocytic pathway, as internalized receptor accumulates in a prevacuolar endosome-like compartment in ren/ mutants. Based on the identity of ren/ with vps2, a class E vps mutant (defective in vacuolar protein sorting), we suggest a model in which the two transport pathways to the vacuole—endocytic transport and the transport of newly synthesized vacuolar enzymes—merge at an intermediate endosomal compartment before vacuolar delivery.

Materials and Methods

Plasmids

pSL552 (Bender and Sprague, 1986) has STE3 under the control of the GAlL promoter carried on YCP50. pSL1922 is the same as pSL552 except for the A365 mutation, a deletion of 310-bp extending from the STE3 Sall site to the Psfl site. In-frame fusion required addition of a 12-bp Sall linker to the Psfl end from which the 3' overhang had been removed by treatment with the Klenow fragment of DNA polymerase. This resulted in the insertion of an arginine codon between codon 365 and the two COOH-terminal STE3 codons.

pSL2099 has the c-myc 9E10 epitope fused to the COOH terminus of GAlL-STE3, carried on the LEU2 CEN/ARS vector pRS315 (Sikorski and Hieter, 1989). 18 new codons, including the c-myc epitope (Evans et al., 1985) were inserted in-frame at the STE3 Psfl site disrupting only the three final STE3 residues; the resulting COOH-terminal 25 residues of this fusion protein are now ENYDGSKMEEQLISEEDFLDRGP (protein sequence from STE3 is underlined).

Strains

SY1793 is a MAfα mfa1Δ mfa2Δ derivative of Sc252 (Whiteway et al., 1990), created by GAlL-HO promoted mating type interconversion and two-step gene replacement at the MFA loci (Table I; Jensen and Herskowitz, 1984; Rothstein, 1991). In addition, the pheromone-dependent FUS1 UAS replaced the HIS3 UAS at the HIS3 locus (Stevenson et al., 1992). The deletion endpoints of mfa1Δ and mfa2Δ are the same as for previously reported disruption alleles, mfa1::LEU2 and mfa2::URA3 (Michaelis and Herskowitz, 1988).

SY1817 is a ste3Δ derivative of SY1793 made by two-step gene replacement. This deletion removes the STE3 coding sequence and UAS, and extends from an Rsal site 417 bp upsteam of the AUC, to a SacI site 111 bp downstream of the stop codon (Hagen et al., 1986). SY1884 is a pep4Δ derivative of SY1817 (Rothman et al., 1986). GAlL-STE3 (SY152) or GAlL-STE3A365 (SY2132) were constructed by two-step gene replacement at the STE3 locus of SY1884.

SY1369 is a Met+ revertant of YY1152 (Clark et al., 1988). ste3Δ (SY1372), GAlL-STE3 (SY1426) and GAlL-STE3A365 (SY1610) alleles were inserted into the chromosome of SY1369 by one step replacement at ste3::URA3, selecting for Ura- derivatives with 5-fluoro-orotic acid (Boeke et al., 1984). SY1683 is a pep4Δ derivative of SY1372 (Rothman et al., 1986). SY1553 is a MAfα version of SY1426 made via an HO-induced mating type switch. SY1616 is a pep4Δ::URA3 derivative of SY1426 made by one step gene replacement (Rothman et al., 1986). SY1498 is a matal derivative of SY1426 carrying Yolh linker insertion mutation 23 (Tatchell et al., 1981).

end1Δ was isolated in a matal cell carrying the MAfα plasmid pSL602 (Bender and Sprague, 1986). Loss of pSL602 yielded SY1534. MAfα (SY1560) and MAfα (SY1614) derivatives of SY1534 were created by HO-promoted mating type interconversion. SY1650 is a MAfα end1Δ segregant derived from SY1614 to SY1426. The GAlL-STE3 constructs in SY1426 and SY1560 were replaced with the natural STE3 promoter by the two step method yielding SY1574 and SY1579, respectively.

SY1675 is MAfα GAlL-STE3 pep4::URA3 segregant from a cross between SY1553 and SY1744 and SY1745 are MAfα STE3 pep4::URA3 and MAfα STE3 segregants of a cross between SY1574 and SY1675.

The GAlL-STE3A365(c-myc) strain SY2559 was constructed by two step gene replacement at the STE3 locus of SY1683. The C-terminus of STE3 A365 construct (described for pSL1922) was tagged at its SalI site with the c-myc epitope (Evan et al., 1985) between Asp 365 and Gly 469. The resulting C-terminus 25 residues of this fusion protein are now YVDGSKMEEQLISEEDFLDRGP (protein sequence from STE3 is underlined).

SY1745 was made GAlL-STE2 via the two step method yielding SY1960;
for this STE2 allele, a 326-bp GALI,10 UAS fragment substitutes for the 360-bp HindIII fragment that includes the natural STE2 UAS. SY2029 is a MATa GALI-STE2 renl-I segregant of a cross between SY1744 and SY1960. SY2041 is a MATa GALI-STE2 renl-I segregant of a cross between SY1579 and SY1960.

**Antiseras**

Rabbit antiserum raised against TrpE-STE3 fusion protein (Clark et al., 1988) was affinity-purified using the fusion protein antigen coupled to CNBr-activated Sepharose 4B (Sigma Immunochemicals, St. Louis, MO). The fusion was purified with the COOH-terminal 183 residues of STE3. Another aliquot of this antiserum was affinity-purified using a TrpE-STE3A365 fusion.

Rabbit antiserum against the STE2 protein was a generous gift from James Konopka (Konopka et al., 1988). Affinity-purified alkaline phosphatase (ALP) rabbit antiserum was generously provided by Chris Raymond (University of Oregon). Mouse mAb-I reactive with the c-myc epitope was purchased from Oncogene Science Inc. (Manhasset, NY).

**Cell Labeling and Immune Precipitation**

Exponential cultures of cells (2 × 10^7/ml) growing at 30°C in supplemented minimal medium lacking methionine and cysteine were pulse-labeled for 10 min with ^[35]S)methionine (0.5 Mci/ml) and then chased with subsequent addition methionine and cysteine to 50 μg/ml. To prepare protein extracts, 250 μl of the labeled cell culture was collected by centrifugation, suspended in 150 μl digestion buffer (DB: 1.4 M sorbitol, 25 mM Tris/Cl pH 7.5, 10 mM sodium azide, 10 mM potassium fluoride, 2 mM MgCl2) with 0.3% β-mercaptoethanol and oxalyticase (Sigma Immunochemicals, St. Louis, MO). The cell suspension was vortexed for 10 min, and 10 μl of the supernatant was added to the supernatant at 1:200. After 1 h at 0°C, IgGSorb was added and incubated at 0°C for 1 h. The immune precipitation buffer (IP; 10 mM Tris/Cl pH 8.0, 0.1% Triton X-100, 2 mM EDTA) with 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 0.5% IgG-Sorb (The Enzyme Center, Maiden, MA). After 15 min at 0°C, IgGSorb was removed by centrifugation and STE3 antiserum was added to the supernatant at 1:1000. After 1 h at 0°C, IgGSorb was added to 0.5%. After 30 min further incubation, immune complexes were washed four times with IP plus 0.1% SDS. Precipitated protein was eluted from the antibody-IgGSorb complex by suspension in SB with 5% SDS and incubation at 100°C for 5 min. The protein was then subjected to SDS-PAGE.

**Immunoblots**

To prepare protein extracts for Western blotting, 1.5 × 10^7 log-phase cells grown at 30°C were pelleted, immediately frozen on dry ice, and then stored at -70°C. Cell pellets were thawed by suspension in 100 μl of SB with 5% SDS and transferred to a 1.5 ml microtube containing an 80 μl volume of glass beads. After being vortexed for 10 min, samples were incubated at 37°C for 10 min. 25 μl of SDS-PAGE sample buffer was added, samples were vortexed for 2 min, and then 10 μl of the supernatant fraction from a 5-min microfuge spin was subjected to electrophoresis. Protein was transferred to nitrocellulose and treated with antibody. For STE3 detection, antiserum that had been affinity-purified against the large TrpE-STE3 fusion was used at 1:100,000. For STE2, antiserum was used at 1:500 dilution. Primary antibodies were detected with an HRP-conjugated anti-rabbit IgG second antibody followed by the ECL chemiluminescent system (Amer sham Corp., Arlington Heights, IL).

**Susceptibility to External Proteases**

At indicated times, 2 × 10^6 cells from a culture pulse labeled with ^[35]S)methionine (as described above) were incubated for 20 min at 0°C with an equal volume of ice-cold medium containing 20 mM potassium fluoride and 20 mM sodium azide. Cells were collected by centrifugation, suspended in 100 μl DB with 0.5% β-mercaptoethanol and incubated at 37°C for 30 min. One half of the sample was treated with protease, with addition of a one-quarter volume of 2,500 U/ml Pronase (in DB) for 60 min at 37°C. The other half was processed identically in parallel except that no protease was added. Protease was removed via two washes of the pelleted cells with 200 μl DB with 1 mM PMSF. Cells were then treated as described above for spheroplasting, extract preparation, and immune precipitation of STE3.

When Western analysis was used to assess susceptibility of receptor to external protease, the procedure was modified in several ways. For each timepoint, 3 × 10^6 cells were collected and prepared as above except that they were incubated in 0.5 ml DB with 0.5% β-mercaptoethanol at 37°C for 30 min before protease treatment. Proteolysis was terminated with the addition of TCA to 17%, samples were frozen on dry ice and then further processed as described (Chaban et al., 1982), except that the TCA-extracted protein was precipitated for 10 min at 0°C, followed by a 10-min microfuge spin at 4°C. The pellet was washed once with acetone, desiccated, and dissolved at 70°C for 5 min in 100 μl SDS-PAGE sample buffer, 10 μl of which was then subjected to SDS-PAGE and immunoblotting.

As a control, accessibility of a cytoplasmic protein, phosphoglucerase kinase (PGK), was assessed with this protocol. No effects of protease treatment were observed. However, when Pronase was added subsequent to spheroplasting and treatment with 1% Triton X-100, PGK was found to be completely degraded.

Cell cultures were treated with a-factor by addition of an equal volume of cell-free filtrate prepared from a saturated culture of EQI23 cells transformed with the a-factor-overproduction plasmid pKK16 (Kocher et al., 1989). Mock a-factor preparations were obtained from the isogenic mfa1::LEU2 mfa2::URA3 strain SM1229.

**Quantitation of Mating**

To quantitate the mating efficiency of various MATa strains after shut-off of receptor synthesis, 10^7 MATa cells (strain 227) were mixed either with 2 × 10^6 MATc cells from a log phase YEPlac20 (2%) culture or with 2 × 10^6 cells from a YEPlac20 culture to which 3% glucose had been added during the final 2 h of growth. The mating mixtures were collected on nitrocellulose filters, and the filters were placed on YEPlac20 (2%) plates or on YEPlacGlucose (2%) plates. After 6 h at 30°C, the number of diploids was titrated by suspending the cells and plating them on medium selective for diploids. Mating efficiency is expressed as the number of diploids divided by the total number of MATa cells present on control filters that did not contain the MATa mate.

**Isolation of ren Mutants**

Cultures from 12 colonies of malal GALI-STE3 strain SY1498 carrying the MATa plasmid pSL602 were treated with either 5.0, 1.5, 0.5% or no ethyl methanal sulfonate for 1 h as described (Moir et al., 1982). Cells were then plated on 2% galactose minimal plates lacking uracil at a density of 3,000 viable cells per plate. Colonies were replica-plated to minimal glucose (2%) plates that had been spread with about 5 × 10^7 MATa cells (strain 227) in 0.3 ml of YEPlac. Colonies that gave a strong mating reaction were picked for further study. Cells mutagenized with 1.5% ethyl methanal sulfonate yielded mutants at a frequency of 1 × 10^{-5}. On the other hand, no mutants were found in a screen of 20,000 unmutagenized cells.

From 10^6 colonies, 70 colonies that exhibited a strong mating reaction were selected. Of these, 45 were confirmed when both mating partners were grown on glucose medium, a protocol that should preclude receptor synthesis. These were presumed to be defective for glucose repression of the GAL promoter and were therefore discarded. To determine whether the remaining mutants carried dominant or recessive mutations, they were crossed to malal strain SY1498, and the mating phenotype of the resulting diploids was assessed by the same protocol used in the original mutant screen. 12 diploids showed strong mating and therefore the corresponding ∆ mutants were presumed to carry dominant mutations. The other 13 mutants carried recessive mutations.

**Indirect Immunofluorescence**

Preparation of fixed, spheroplasted cells for indirect immunofluorescence was carried out essentially as described (Roberts et al., 1991), except that oxalyticase at a final concentration of 20 μg/ml was used for spheroplasting.

For detection of the receptor with the STE3 antiserum, antibodies that had been affinity-purified against the truncated fusion protein TrpE-STE3Δ365 were used. These should react equally well with the STE3A3636 receptor as they do with the wild-type protein. After incubation with the STE3 antibodies, used at a 1:120 dilution, signal was further amplified through three subsequent incubations with secondary antibodies (purchased from Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at 2 μg/ml: (α) goat anti-rabbit IgG; (β) rabbit anti-goat IgG; and (γ) fluorescein-anti goat-anti rabbit IgG. Antibody incubations were 1 h at room temperature.

For the double-staining of mcs-tagged receptor and ALP, mouse mAb-I (Oncogene Science Inc.) was used at a dilution of 1:20, while the rabbit anti-
Strains designated "A" in the reference column are isogenic to SY1369, except as indicated. Strains designated "B" are isogenic to SY1793.

ALP antiserum, which had previously been affinity purified and adsorbed against cells deleted for the ALP structural gene (pho8Δ), was used at 1:10. Detection of the myc antibody involved addition of 5 μg/ml biotinylated goat anti-mouse, followed by 5 μg/ml fluoresceinated streptavidin. Visualization of the anti-ALP antibodies was achieved via addition of 5 μg/ml rhodamine-conjugated goat anti-rabbit secondary antibodies.

Results

Rapid, Ligand-independent Endocytosis Delivers the α-factor Receptor to the Vacuole

As noted in the introduction, analysis of the abundance of were prepared and treated with STE3 antiserum. Immunoprecipitates were subjected to SDS-PAGE and STE3 was visualized by autoradiography. (B) MATα pep4::URA3 cells (strain SY1744) were labeled for 10 min with [35S]methionine. Samples were taken 1, 10, 60, and 90 min after initiation of the chase. Cells were digested with 1 mg/ml Pronase (+), or mock digested with no protease (−) for 1 h at 37°C. Extracts were prepared and treated as described above. The upper panel shows immune precipitated full-length STE3. The panel below shows an over-exposed portion of the same gel, with the arrow indicating the position of the 30-kD COOH-terminal STE3 digestion product.

Figure 1. Turnover and surface accessibility of the α-factor receptor. (A) A wild-type MATα strain (SY1574) and its isogenic pep4::URA3 derivative (SY1744) were labeled for 10 min with [35S]methionine as described in Materials and Methods. Samples were taken 2, 15, 30, and 60 min after initiation of the chase, and then extracts were prepared and treated with STE3 antiserum. Immunoprecipitates were subjected to SDS-PAGE and STE3 was visualized by autoradiography. (B) MATα pep4::URA3 cells (strain SY1744) were labeled for 10 min with [35S]methionine. Samples were taken 1, 10, 60, and 90 min after initiation of the chase. Cells were digested with 1 mg/ml Pronase (+), or mock digested with no protease (−) for 1 h at 37°C. Extracts were prepared and treated as described above. The upper panel shows immune precipitated full-length STE3. The panel below shows an over-exposed portion of the same gel, with the arrow indicating the position of the 30-kD COOH-terminal STE3 digestion product.
surface binding sites suggests that the yeast α-factor receptor is subject to ligand-mediated endocytosis (Jenness and Spatrick, 1986; Chvatchko et al., 1986). To analyze receptor fate directly in both the absence and presence of pheromone, we developed antibodies that recognize the COOH-terminal hydrophilic domain of the α-factor receptor, STE3 (Clark et al., 1988).

We first examined the stability of STE3 protein in the absence of added α-factor in wild-type cells, as well as in pep4Δ cells, which are deficient for vacuolar protease activity. STE3 protein was labeled with [35S]methionine in a pulse-chase protocol, immunoprecipitated from cell extracts, and subjected to polyacrylamide gel electrophoresis. As shown in Fig. 1A, STE3 was unstable in wild-type cells, exhibiting a half-life of ~20 min. In contrast, the receptor was extremely stable in the pep4Δ background, showing a half-life much greater than 2 h. Because of the possibility that a culture of α cells could contain low levels of α-factor (for instance, due to rare mating type switches) we also examined STE3 protein turnover in MATα pep4Δ cells deleted for the α-factor structural genes MFA1 and MFA2. In these cells, the kinetics of STE3 turnover were identical to wild-type cells, indicating that turnover is indeed ligand independent (data not shown).

The rapid rate of receptor degradation and the dependence of this degradation on vacuolar proteases suggests that newly synthesized receptor is delivered to the cell surface, but resides there only a short time before being internalized and...

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delivered to the vacuole for degradation. As one test of this interpretation, we labeled receptor by a pulse-chase protocol and measured its susceptibility to digestion when whole cells were treated with exogenous protease (Trueheart and Fink, 1989). pep4Δ cells were used to preclude endogenous turnover of the receptor. As seen in Fig. 1 B, a small fraction of the STE3 protein, labeled during a 10-min [35S]methionine pulse, was susceptible to protease after 1 min of chase. This susceptibility is revealed both by a reduction in the amount of full-length (48 kD) protein and by the appearance of a 30-kD digestion product. The mass of this product, coupled with its reactivity to the STE3 antiserum, implies that it corresponds to the seventh transmembrane segment and the COOH-terminal cytoplasmic domain. After 10 min of chase, ~70% of the radio-labeled receptor is susceptible to proteolysis by exogenous protease. We interpret these results to mean that only a small fraction of receptor synthesized during the 10-min pulse has been delivered to the cell surface by 1 min into the chase period but that the majority of the newly synthesized receptor is at the cell surface 10 min into the chase period. At longer chase times, the radio-labeled receptor is resistant to exogenous protease, suggesting that receptor that had been at the surface has been internalized (Fig. 1 B, 60- and 90-min timepoints). This internalization requires energy metabolism, as receptor remains susceptible to protease if potassium fluoride and so- externalization requires energy metabolism, as receptor re-
ceptible to protease after 1 h of chase. This is 40% of the receptor-derived fluorescence signal is lost from the surface of MATtot seclΔ cells. As a further test of the conclusion that the a-factor receptor is routed to the vacuole via endocytosis from the cell surface, we examined the effect of the seclΔ mutation on receptor location. At the restrictive temperature, delivery of newly synthesized proteins to the surface is blocked in seclΔ cells; surface-targeted proteins instead accumulate in secretory vesicles (Novick and Schekman, 1979). In contrast, delivery of proteins whose primary destination is the vacuole proceeds normally in seclΔ cells (Stevens et al., 1982; Roberts et al., 1989). After temperature shift of MATα pep4Δ seclΔ cells, expression of a GALI-driven c-myc-tagged STE3 protein was induced with galactose and receptor visualized by immunofluorescence. No vacuolar staining was seen (data not shown). Instead dispersed punctate cytoplasmic staining was found often concentrated near the growing end of the cell (data not shown). Thus, delivery of receptor to the vacuole requires prior delivery to the cell surface.

Mammalian cell surface receptors that undergo constitutive,

Figure 3. Turnover of the COOH-terminal Truncated Receptor. MATα steΔ cells (SY1817) carrying GALI-ST3 (pSL552) or GALI-ST3Δ365 (pSL922) were grown to log phase on galactose medium. Receptor synthesis was shut off with the addition of 3% glucose. Samples were taken just before glucose addition and at 1 and 2 h after glucose addition. STE3 was visualized by Western analysis of extracts. Molecular weight standards (in kilodaltons) are indicated at right.

Figure 4. Susceptibility of the COOH-terminal truncated receptor to exogenous protease. MATα pep4Δ::URA3 cells that were GALI-ST3 (SY1616) or GALI-ST3Δ365 (SY1684) were grown for 2.5 h in the presence of 2% galactose, 3% glucose was added, and growth continued for 2.5 h. Whole cells were digested with 1 mg/ml Pronase (+), or mock digested with no protease (−) for 1 h at 37°C. Subsequently, extracts were prepared and subjected to Western analysis using STE3 antiserum. Molecular weight standards (in kilodaltons) are indicated at right.

Receptor COOH-terminal Truncation Mutants Are Disabled for Constitutive Endocytosis

Mammalian cell surface receptors that undergo constitutive,
ligand-independent endocytosis have been found to have sequences in their cytoplasmic domains that direct sequestration into clathrin coated pits and subsequent internalization (Trowbridge, 1991). Receptor mutants deleted for these "endocytosis signals" do not get internalized. To examine the role of STE3 protein's 187-residue COOH-terminal cytoplasmic domain in receptor endocytosis, we constructed STE3 mutants partially truncated for this domain. These mutants retain the ability to function in signal transduction as they fully complemented ste3Δ alleles. The receptor structural genes were placed under control of the inducible GALI promoter and the ability of the mutant receptors to be internalized was evaluated by the same assays used above for wild-type receptor: PEP4-dependent turnover, accessibility to surface protease, and indirect immunofluorescence.

To follow turnover, cells with either wild-type or mutant receptor GALI constructs were grown on galactose, to allow expression, and then glucose was added to block further synthesis. At intervals, samples were withdrawn and STE3 abundance was assessed by Western analysis of cell extracts. As with the radioactive pulse-chase experiment, wild-type STE3 was rapidly turned over (Fig. 3). STE3ΔA33, missing the COOH-terminal 37 residues, showed a stability similar to wild-type receptor (data not shown). In contrast, STE3ΔA399 and STE3ΔA365, missing 71 and 105 residues, were very stable and showed no degradation two hours after the block to new synthesis (Fig. 3 and data not shown). The degradation of STE3 protein therefore requires a segment of the COOH-terminal domain.

Accessibility of receptor to exogenous protease was assessed in pep4Δ cells having either the GALI-STE3 or the GALI-STE3ΔA365 construct. Cells were grown in galactose for 2.5 h, glucose was added to halt further synthesis, and protease treatment was initiated after a 2.5-h incubation in the glucose medium. As seen previously with radiolabeled STE3 (Fig. 1B), wild-type receptor accumulated in a protease-resistant compartment (Fig. 4), presumably the vacuole. The protease susceptibility of STE3ΔA365 protein was markedly different from wild-type STE3. Even 2.5 h after imposition of the glucose block to synthesis all truncated receptor was sensitive to external protease, as revealed by the disappearance of the 33-kD STE3ΔA365 band and the appearance of the 18-kD digestion product.

Finally, by indirect immunofluorescence microscopy, the STE3ΔA365 protein accumulated at the cell surface (Fig. 5), whereas wild-type receptor accumulated in the vacuole, as before (Fig. 2). The surface accumulation of STE3ΔA365 does not result from its overproduction from the GALI promoter. When expressed from its natural promoter, STE3ΔA365 was stable and exclusively located at the cell surface (data not shown). We conclude that the STE3ΔA365 receptor is not subject to constitutive endocytosis.

**Ligand-dependent Receptor Internalization**

The experiments described thus far establish that the a-factor receptor is internalized continuously in the absence of ligand. Does the receptor also exhibit ligand-induced endocytosis? To answer this question we took advantage of STE3ΔA365, which is disabled for constitutive endocytosis. Synthesis of receptor was induced by growth of GALI-STE3ΔA365 pep4Δ cells in galactose for 90 min and receptor was allowed to accumulate at the cell surface during a 60-min glucose chase. The location of the receptor was evaluated by the extracellular protease assay at various times after addition of a-factor. In the absence of added a-factor, STE3ΔA365 was susceptible to digestion by external protease (Fig. 6). In this experiment the cytoplasmic domain digestion product was run off the gel, so protease susceptibility is manifest only by the disappearance of the receptor protein. 10 min after a-factor addition, a substantial portion of the receptor was resistant to protease, and after 90 min, the receptor protein was completely resistant to protease. We conclude that pheromone caused the receptor to be removed from the surface and delivered to an internal cellular compartment. Thus, although STE3ΔA365 is totally disabled for constitutive endocytosis, it remains capable of a pheromone-dependent internalization.

![Figure 6. Susceptibility of the COOH-terminal truncated receptor to exogenous protease after treatment of cells with pheromone. MATα pep4Δ GALI-STE3 ΔA365 pep4Δ cells (SY2132) were grown for 1.5 h in the presence of 2% galactose, chased for 1 h with 3% glucose, and then treated with pheromone or mock pheromone. At the times indicated, samples were removed and 10 mM sodium azide was added. Whole cells were then subjected to surface proteolysis with 1 mg/ml Pronase (+) for 1 h at 37°C or mock digested (-) with no protease. STE3ΔA365 protein was visualized by Western analysis.](image-url)
Correlated with the change in subcellular location, pheromone also caused extensive covalent modification of the receptor protein, as seen in Fig. 6. Although we have not investigated the nature of this modification, it is known that other members of this receptor family, including the α-factor receptor, show increased phosphorylation on the COOH-terminal domain in response to hormone (Reneke et al., 1986; Dohlman et al., 1991).

α-factor–dependent internalization was also followed by indirect immunofluorescence microscopy. For this experiment, the STE3Δ365 receptor was tagged at its COOH terminus with the c-myc 9E10 epitope (Evan et al., 1985) and placed under the control of the GAL1 promoter. These cells were cultured via the galactose-glucose protocol and then treated with α-factor or mock pheromone for 1.5 h. The mock-treated cells primarily showed surface staining (Fig. 7). In contrast, after α-factor treatment no surface staining was evident. Instead all of the receptor-derived fluorescence signal was seen to be vacuolar, showing complete coincidence with the ALP-derived fluorescence and with the vacuolar depolarization seen by Nomarski microscopy (Fig. 7).

Constitutive and Ligand-dependent Endocytosis Are both G Protein-independent Processes

The endocytic uptake of α-factor pheromone and the correlated loss of surface binding sites was found to proceed upon pheromone challenge in MATα cells lacking the heterotrimeric G protein (Jenness and Spatrick, 1986; Zanolari et al., 1992). To determine whether the constitutive or ligand-mediated endocytosis of the α-factor receptor requires the G protein, we expressed GAL1–STE3 or GAL1–STE3Δ365 receptor constructs in diploid cells, which do not transcribe the G protein structural genes (Miyajima et al., 1987; Dietzel and Kurjan, 1987; Whiteway et al., 1989). Both constitutive and ligand-dependent endocytosis proceeded with normal kinetics (data not shown). This implies that the endocytic machinery recognizes the receptor proper, not a G protein/receptor complex.

Genetic Screen for Trans-acting Mutants that Block Endocytic Turnover of STE3 Protein

The different locations of wild-type STE3 and STE3Δ365 proteins following the galactose to glucose pulse–chase protocol might result in different mating capacities for strains carrying these constructs. We therefore compared the ability of GAL1–STE3 and GAL1–STE3Δ365 strains to mate under several conditions. When growth and mating were carried out on galactose medium, which allows continuous receptor synthesis, the strains mated equally well (Table II). However, when cells were grown on galactose but transferred to glucose medium 2 h before mating, GAL1–STE3Δ365 cells mated efficiently, but GAL1–STE3 cells mated poorly (Table II). The ability of the STE3Δ365 cells to mate appears to reflect the retention of receptor at the surface rather than simply the failure to degrade the receptor, as GAL1–STE3 pep4Δ cells mated poorly even though the receptor is stable (Table I).

We took advantage of the mating difference of GAL1–STE3 and GAL1–STE3Δ365 to isolate mutants disabled for receptor internalization. Mutagenized GAL1–STE3 cells were screened for mutants that showed strong mating following glucose-mediated repression of new receptor synthesis, and 25 mutant colonies were identified (see Materials and Methods). To determine whether the mutations were dominant or recessive, the mutants were mated to a mata/ strain and the mating properties of the resulting α/α− diploid assessed in the galactose/glucose protocol (note: α/α− diploids mate as α cells; Kassir and Simchen, 1976). By this criterion 12 of the 25 mutants carried dominant mutations. Of these, four are likely chain termination mutations in STE3 because immunoblotting revealed that they expressed truncated forms of STE3 protein. The other eight dominant mutations may be STE3 alleles that lead to more subtle alterations in STE3 or they may be mutant alleles of other genes.

Analysis of ren1-1 Mutants

The recessive mutations should identify trans-acting functions required for endocytosis. We chose a particularly strong example of this class for further study. This mutation segregated as a single gene unlinked to STE3. As the mutant is defective in receptor endocytosis (see below), the gene has been named REN1. We quantitated the mating of ren1-1 cells in the galactose-glucose mating protocol used to isolate the mutant. As shown in Table II, ren1-1 cells mated as well as GAL1–STE3Δ365 cells. Northern blot analysis showed that ren1-1 does not affect the synthesis or stability of STE3 mRNA. Moreover, glucose repression of GAL1–STE3 mRNA synthesis occurred with identical kinetics in wild-type and ren1-1 cells (data not shown). We therefore considered it likely that ren1-1 affected receptor endocytosis and tested the possibility directly.

Table II. Mating Capacity of Mutants Defective for Endocytosis or Turnover of STE3 Protein

| Strain       | Relevant genotype | Efficiency of mating | Gal-to-Gal | Gal-to-Glu |
|--------------|-------------------|----------------------|------------|------------|
| SY1426       | GAL1–STE3         | 0.9                  | 0.0003     |            |
| SY1610       | GAL1–STE3Δ365     | 0.8                  | 0.3        |            |
| SY1616       | GAL1–STE3 pep4::URA3 | 0.9              | 0.001      |            |
| SY1650       | GAL1–STE3 ren1-1  | 0.9                  | 0.3        |            |

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This mobility shift is most readily observed in slowed relative to its mobility when extracted from therefore believe that it reflects a modification that occurs in the vacuole where the receptor accumulates. Molecular weight stand-

dards (in kilodaltons) are indicated at right. We examined the turnover of wild-type STE3 protein in both
tants. Ligand-mediated turnover of STE3A365 was also
tutive turnover of wild-type STE3 is slowed in
chase protocol. In wild-type cells, receptor turnover was
with [3~S]methionine, STE3 expressed from its own pro-
abundant in
rooter exhibited a half-life of ~90 rain in the
ExtraceUular Protease
STE3 Protein of renl-1 Cells Is Susceptible To
the location of STE3 protein in
lar occupies a novel compartment. By immunofluorescence
microscopy, it is found to stain bodies located just adjacent to the vacuole. Such bodies may
represent an intermediate endocytic compartment through
which the receptor normally passes on its way from surface to vacuole.

renl-1 Slows STE3 Turnover

We examined the turnover of wild-type STE3 protein in both REN1+ and renl-1 cells using the galactose-glucose pulse-
chase protocol. In wild-type cells, receptor turnover was rapid. In renl-1 cells, however, turnover was slowed considerably (Fig. 8 A). In separate pulse-chase experiments with [35S]methionine, STE3 expressed from its own pro-
moter exhibited a half-life of ~90 min in the renl-1 back-
ground (data not shown), compared to 20 min in REN1+ cells (Fig. 1). Mutations that slow STE3 protein turnover should lead to steady state over-accumulation of the receptor. In keeping with this expectation, STE3 protein ex-
pressed from its natural promoter was found to be more abundant in renl-1 cells than in wild-type cells (Fig. 8 B).

The experiments described above established that constitu-
tive turnover of wild-type STE3 is slowed in renl-1 mu-
tants. Ligand-mediated turnover of STE3A365 was also slowed in renl-1 cells (data not shown).

STE3 Protein of renl-1 Cells Is Susceptible To
Extracellular Protease

The mating and receptor turnover phenotypes of the renl-1 mutant are very similar to those of cells expressing STE3A365 protein. We were therefore interested to compare the location of STE3 protein in renl-1 cells to its location in wild-type cells. We first used the extracellular protease assay and the GAL1 constructs to evaluate receptor location after a galactose to glucose pulse-chase protocol. As expected, protease caused essentially no diminution of the full-length wild-type STE3 protein expressed in wild-type cells, and only a small amount of released cytoplasmic tail digestion product was observed (Fig. 9). Under the same conditions, all of the STE3A365 protein was susceptible to the protease and was converted to its corresponding cytoplasmic domain digestion product. The result for renl-1 cells is intermediate between these two extremes. In the renl-1 background, STE3 protein was partially susceptible to the external protease, and a substantial amount of the cytoplasmic tail digestion product was seen (Fig. 9). However, in contrast to the results seen for STE3Δ365, a substantial fraction of total STE3 protein was resistant to protease.

Internalized STE3 Is Extra-vacuolar in renl-1 Cells

The experiments described above imply that constitutive end-
cytosis may be impaired, but not completely blocked, in the renl-1 background; some receptor clearly is internalized by the mutant cells. To determine the location of this internal-
ized receptor we stained mutant and wild-type cells with antibodies that recognize the myc-tagged STE3 protein and antibodies that recognize the vacuolar membrane protein, ALP. 30 min after glucose-mediated shut off of the myc-
tagged GAL1-STE3 renl-1, but not REN1+ cells, retained a significant amount of receptor at the cell surface (Fig. 10). Both genotypes also exhibited internal staining for receptor, but the pattern of staining was markedly different. In the REN1+ background, internalized receptor was vacuolar. Receptor staining showed complete colocalization with the immunofluorescent signal from the vacuolar marker protein ALP. Furthermore both signals coincided with vacuolar depressions seen by Nomarski microscopy. Most renl-1 cells, on the other hand, showed little or no overlap of the two signals. Instead, bodies staining brightly for the receptor were often seen adjacent to the vacuole. Such bodies may represent an intermediate endocytic compartment through which the receptor normally passes on its way from surface to vacuole.

Similar extra-vacuolar bodies have recently been de-
scribed for a subset of mutants defective in vacuolar protein sorting, Class E vps mutants (Raymond et al., 1992). vps mutants were selected for their aberrant secretion to the culture medium of the soluble vacular protease, carboxypepti-
dase Y (CPY) (Bankaitis et al., 1986; Rothman and Stevens, 1986). The Class E mutant subset secretes ~40% of newly synthesized CPY. In addition, CPY that remains intracellular occupies a novel compartment. By immunofluorescence microscopy, it is found to stain bodies located just adjacent to the vacuole. These structures also contain several other vacuolar membrane proteins whose vacuolar delivery is also presumably blocked (Raymond et al., 1992).

Because of this morphological similarity of renl cells with Class E vps mutants, we asked if renl showed a vps sorting defect for CPY. Indeed, like the Class E vps mutants, renl-1 cells were shown to secrete some CPY (data not shown).
Given that renl-1 cells exhibit a weak vps" phenotype, we
considered the possibility that renl-1 may be an allele of a
known VPS gene. Complementation analysis of renl-1 with
the complete set of vps mutants indicated an identity with
vps2, a member of the Class E subset (Raymond et al.,
1992).

**Turnover of the α-factor Receptor Is**
**REN1- and PEP4-dependent**

To extend the analysis of receptor turnover, we examined the
metabolism of the other pheromone receptor, the α-factor
receptor (STE2), in a variety of genetic backgrounds using a
GAL1–STE2 construct. Like STE3 protein, STE2 protein
was degraded rapidly in wild-type cells during the glucose
chase. The renl-1 mutation slowed this turnover, and pep4Δ
blocked turnover altogether (Fig. 11).

**Discussion**

**Pheromone Receptors Are Subject to Two
Modes of Endocytosis**

Two distinct modes of endocytosis appear to operate on the
yeast α-factor receptor: a constitutive, ligand-independent
mechanism and a regulated, ligand-dependent mechanism.
In both types of endocytosis the receptor traverses the same
subcellular territory, moving from the cell surface to the
vacuole where it is degraded.

Three experimental observations established that the
α-factor receptor is subject to endocytosis in the absence of
ligand. First, pulse-chase experiments demonstrate that the
receptor is an unstable protein whose degradation depends
on vacuolar proteases. Second, using exogenous protease to
measure the amount of receptor present at the cell surface,
we found that newly synthesized α-factor receptor remains
protease sensitive only for a short interval after its delivery
to the surface, implying a rapid constitutive internalization.
Finally, indirect immunofluorescence assays show that over
the same time course the receptor accumulates in the vacuole.
Together our results provide the following picture of the
dynamics of receptor trafficking. Newly synthesized recep-
tor is delivered via the secretory pathway to the cell surface
where it resides only transiently, exhibiting a half-life of
~20 min. Receptor is then internalized and delivered to the
vacuole where it is degraded.

A second mode of endocytosis was recognized because of
the properties of α-factor receptor mutants lacking half of the
COOH-terminal cytoplasmic domain. These mutants fail to
undergo constitutive endocytosis, suggesting that the STE3
Δ365 and STE3A399 mutations delete "signals" on the recep-
tor necessary to this process. For example, perhaps these
signals are required for capture into clathrin-coated pits (see
below). Despite the block to constitutive endocytosis, these
mutant receptors are still subject to ligand-mediated endocy-
tosis. Because the mutant receptors are defective for one
mode of endocytosis but not the other, we suggest that there
is at least one mechanistic step that distinguishes the two
classes of endocytosis. Another possibility is that the en-
docytosis machinery recognizes a holistic feature of the
COOH-terminal cytoplasmic domain, perhaps the extent of
a posttranslational modification. In this view, the COOH-
terminal receptor mutants after pheromone treatment would
show the same extent of modification as wild-type receptor
exhibits in the absence of pheromone.

The α-factor receptor also exhibits both modes of endocy-
tosis. By assays identical to those used to analyze the α-factor
receptor, we showed that the α-factor receptor is subject to
rapid constitutive endocytosis. Previous studies implied
that the α-factor receptor was also subject to ligand-
mediated endocytosis. In particular, it was found that when
cells were treated with pheromone, cell surface binding sites
were lost rapidly (half-life ~20 min) (Jenness and Spatrick,
1986; Konopka et al., 1988; Reneke et al., 1988), presum-
ably reflecting endocytosis of the receptor. Although not the
focus of these studies, the experiments also provided hints
that the α-factor receptor undergoes constitutive endocyto-
sis. In particular, a slow loss of α-factor binding sites was
observed in the absence of ligand (surface half-life >2 h at
25°C; about 45 min at 34°C). This rate differs considerably
from the rapid turnover we observe for both pheromone
receptors and likely reflects the different protocol used (their
experiments were carried out in the presence of cyclohexi-
mide at 25 or 34°C). Nonetheless, it seems likely that the
two experimental protocols reveal the same process, as
α-factor receptors deleted for part of the COOH-terminal cy-
toplasmic domain were blocked for this slow constitutive
loss of binding sites, but remained competent for the ligand-
mediated loss (Konopka et al., 1988). If this interpretation
is correct, these data again imply that constitutive and
ligand-mediated endocytosis may have a different mechanis-
tic basis.

The rapid constitutive endocytosis of the pheromone
receptors is not likely due to bulk endocytosis of the plasma
membrane. Rather, two observations imply that the capacity
for constitutive endocytosis is specifically built in to these
proteins. First, the COOH-terminally truncated receptors,
STE3A365 and STE3A399 fail to undergo constitutive en-
docytosis presumably because they lack requisite signals.
Second, the plasma membrane ATPase is turned over very
slowly, showing a half-life of >10 h (Benito et al., 1991).

**Possible Roles for Constitutive Endocytosis**

Constitutive endocytosis of the pheromone receptors seems
at first glance to be an unproductive and unnecessary pro-
cess. What purpose might it serve? Two possibilities are es-
pecially appealing. First, receptor endocytosis may facilitate
the switch in receptor type that must occur when yeast cells
undergo mating-type interconversion. Most wild strains of
yeast are homothallic and under certain conditions can
change mating types as frequently as every cell division cy-
kle. The change is effected by a switch in the genetic informa-
tion present at the mating-type locus, which then directs the
synthesis of the receptor and pheromone species appropriate
for the new mating type. This rapid phenotypic conversion
of mating type likely requires removal of old receptor be-
cause cells that express both receptors are defective for mat-
ing (Bender and Sprague, 1989). Constitutive endocytosis
provides a rapid means to achieve removal of old receptor.

A second role for constitutive endocytosis may be in part-
ner selection during the mating process. a and α cells sense
the location of nearby potential mating partners, apparently
by detecting a gradient of pheromone concentration (Jackson
and Hartwell, 1990a,b). In response, they reorient cell
polarity and focus growth toward the partner. Newly synthesized receptor is therefore deposited at the region of the cell surface nearest the partner. The simultaneous removal of receptor from other surface sites by constitutive endocytosis would reinforce the emerging asymmetric receptor distribution. Thus, constitutive endocytosis may be an important component of partner selection during the courtship phase of mating. In keeping with this possibility, strains expressing COOH-terminal truncated receptors are impaired for partner selection (Jackson and Hartwell, 1990a; C. Boone, N. Davis, and G. Sprague, unpublished results).

In principle, constitutive endocytosis could also serve as a means to set the cell's level of sensitivity to pheromone. COOH-terminal truncated receptors are defective for constitutive endocytosis and also confer increased sensitivity to pheromone (Konopka et al., 1988; Reneke et al., 1988; C. Boone, N. Davis and G. Sprague, unpublished results). However, we infer that the correspondence of these two receptor properties is fortuitous because renl mutants, which are impaired for constitutive endocytosis of wild-type receptor, do not show increased sensitivity to pheromone (N. Davis and G. Sprague, unpublished results). Thus, it seems unlikely that constitutive endocytosis plays a major role in determining sensitivity to pheromone.

Functions Required for Endocytosis

To begin to identify gene products required for endocytosis we developed a screen to isolate mutants defective for the process. The screen uses a simple mating assay to reveal mutants that retain functional receptor at the surface after a shut off of new receptor synthesis. In principle, mutants may be blocked either at the initial stages of endocytosis or at any stage from which endocytic vesicles can recycle to the surface. In this context, it should be noted that receptors located in the vacuole cannot be recycled to the surface. This conclusion follows from the observation that pep4 mutants, which accumulate receptor in the vacuole, cannot mate in the assay (Table II). Thus, the mutant isolation scheme will not reveal functions that are simply required for vacuolar proteolytic activity. The scheme requires that the mutants be defective for constitutive endocytosis, but they may also be defective for ligand-mediated endocytosis if the two processes share common steps.

Analysis of one mutant strain revealed that constitutive endocytosis is indeed altered. In this renl strain, receptor accumulates in two locations—at the cell surface and in an intracellular compartment that lies near the vacuole. We suggest that this compartment represents a yeast endosome. The renl defect apparently blocks traffic from the putative endosome to the vacuole. In animal cells, the endosome serves a crossroads where the fate of internalized material is decided—whether to continue on to the lysosome for degradation or to exit the pathway, as many receptors do, and recycle back to the cell surface. If recycling occurs in yeast, then the accumulation of receptor at the surface of renl mutant cells could be a secondary result of a renl-imposed block to vacuolar delivery. Receptor that accumulates in the endosome may be free to recycle back to the surface via an existing recycling pathway. Alternatively, the accumulation of receptor at the surface may indicate that REN1 has a direct role in receptor internalization. Although our analysis of the renl-1 mutant has focused on constitutive endocytosis, the observation that ligand mediated turnover of receptor (STE3Δ365) is slowed in renl-1 cells indicates that REN1 is also required for ligand mediated endocytosis.

The finding that REN1 is identical to VPS2 implies that this gene is required for proper function of two modes of transport to the vacuole—transport of proteins internalized by endocytosis and the transport of newly synthesized vacuolar enzymes. One possibility is that these two pathways converge at a point before delivery to the vacuole and that REN1/VPS2 is required to carry out a step after the convergence. Indeed, in animal cells, a similar convergence of lysosome biogenetic and endocytic pathways has been established (Griffiths et al., 1988). In keeping with this possibility, Class E vps mutants, including vps2, accumulate a novel organelle that contains a number of newly synthesized proteins normally destined for vacuolar delivery: CPY, the vacuolar ATPase, and di- and dipeptidyl peptidase B (Raymond et al., 1992). (We note, however, that not all vacuolar proteins—in particular ALP—are impaired for delivery to the vacuole; for discussion see Raymond et al., 1992.) These organelles are likely identical to those containing internalized a-factor receptor that we observe in the renl mutants. Proof of this will await double-stained immunofluorescence showing colocalization of endocytosed receptor and vacuolar proteins within these presumptive endosomes.

Although not identified by our small collection of Ren- mutants, clathrin appears to be a second function required for constitutive endocytosis. As noted in the introduction, disruption of the clathrin heavy chain gene leads to a reduction in the rate of a-factor uptake (Payne et al., 1988). Moreover, we find that a temperature-sensitive mutation in this gene leads to a reduction both in the rate of a-factor uptake and in the constitutive internalization of the a-factor receptor upon shift to nonpermissive temperature (P. Tan, N. Davis, G. Sprague, and G. Payne, unpublished results). The rapid onset of these phenotypes after temperature shift implies a direct role for clathrin in the endocytosis of the yeast pheromone receptors.

Recently, three newly identified genes have been suggested to have a role in the endocytosis of the yeast pheromone receptors (Wichmann et al., 1992; Rath et al., 1993). Mutation of these genes results in a defect in the uptake and/or degradation of the a-factor pheromone. end3 and end4 mutant cells display surface binding sites for a-factor pheromone but are defective for pheromone uptake, implying that they are defective for the initial step of receptor internalization from the surface (Raths et al., 1993). On the other hand, the yeast YPT7 gene, isolated by virtue of homology to Rab7, which encodes a late endosome-associated GTP-binding protein in animal cells, may control a later step in the endocytic pathway. Disruption of YPT7 blocks a-factor degradation, but not the initial uptake of pheromone by the mutant cells (Wichmann et al., 1992). However, the defect in a-factor degradation associated with ypt7 mutants may not be due to impaired endocytic transport. Instead, it may reflect impaired vacuolar function. Indeed, ypt7 mutant cells show a generalized defect in the processing of vacuolar zymogens as well as a grossly disrupted vacuolar morphology (Wichmann et al., 1992).

Our analysis of the endocytosis of the yeast pheromone receptors has begun to define discrete steps in a pathway for receptor transport connecting the cell surface to the vacuole.
The finding that the endocytic and vacuolar biogenic pathways likely converge in yeast, just as they do in animal cells, coupled with the involvement of clathrin and the possible involvement of Rab proteins in both the yeast and animal cell processes (Chavrier et al., 1990; van der Sluis et al., 1991, 1992; Buccioni et al., 1992; Wichmann et al., 1992), implies that many functions required for endocytosis in yeast will have animal cell counterparts. Analysis of additional Ren mutants should identify such functions.

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