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Elimination of Defective α-Factor Pheromone Receptors

DUANE D. JENNESS,* YU LI, CHRISTOPHER TIPPER, AND PHYLLIS SPATRICK

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655-0122

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This report compares trafficking routes of a plasma membrane protein that was misfolded either during its synthesis or after it had reached the cell surface. A temperature-sensitive mutant form of the yeast α-factor pheromone receptor (*ste2-3*) was found to provide a model substrate for quality control of plasma membrane proteins. We show for the first time that a misfolded membrane protein is recognized at the cell surface and rapidly removed. When the *ste2-3* mutant cells were cultured continuously at 34°C, the mutant receptor protein (Ste2-3p) failed to accumulate at the plasma membrane and was degraded with a half-life of 4 min, compared with a half-life of 33 min for wild-type receptor protein (Ste2p). Degradation of both Ste2-3p and Ste2p required the vacuolar proteolytic activities controlled by the *PEP4* gene. At 34°C, Ste2-3p comigrated with glycosylated Ste2p on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that Ste2-3p enters the secretory pathway. Degradation of Ste2-3p did not require delivery to the plasma membrane as the *sec1* mutation failed to block rapid turnover. Truncation of the C-terminal cytoplasmic domain of the mutant receptors did not permit accumulation at the plasma membrane; thus, the endocytic signals contained in this domain are unnecessary for intracellular retention. In the *pep4* mutant, Ste2-3p accumulated as series of high-molecular-weight species, suggesting a potential role for ubiquitin in the elimination process. When *ste2-3* mutant cells were cultured continuously at 22°C, Ste2-3p accumulated in the plasma membrane. When the 22°C culture was shifted to 34°C, Ste2-3p was removed from the plasma membrane and degraded by a *PEP4*-dependent mechanism with a 24-min half-life; the wild-type Ste2p displayed a 72-min half-life. Thus, structural defects in Ste2-3p synthesized at 34°C are recognized in transit to the plasma membrane, leading to rapid degradation, and Ste2-3p that is preassembled at the plasma membrane is also removed and degraded following a shift to 34°C.

The plasma membrane separates the interior of the cell from its extracellular environment. Integral membrane proteins play vital roles in the function of the plasma membrane by mediating such processes as nutrient transport, maintenance of electrochemical gradients, and transduction of sensory information. Monitoring and maintaining the structural integrity of these proteins present special problems for the cell, since the proteins are exposed continuously to the external environment yet their function is monitored largely inside the cell. Specific signals are known to result in the elimination of plasma membrane proteins from the cell surface, that is, endocytosis in response to specific ligands or when the metabolic or developmental state of the cell has been altered (61). Degradation of defective membrane proteins in the endoplasmic reticulum (ER) has also been described (8, 26, 46, 63). However, little is known about the fate of membrane proteins that suffer damage after they have been inserted into the plasma membrane. In principle, temperature-sensitive mutants are powerful tools for monitoring the elimination of such proteins, because the structural defect can be induced after the protein has reached the plasma membrane. This report describes the fate of a temperature-sensitive form of the yeast α-factor receptor.

The α-factor pheromone receptor, encoded by the *STE2* gene, is a G-protein-coupled receptor that is required for the mating of the **a** and α haploid cell types of the yeast *Saccharomyces cerevisiae* (reviewed in references 4, 59, and 69). The tridecapeptide α-factor is secreted by haploid α cells and binds to receptors on the surface of haploid **a** cells. The **a** cells respond to α-factor by arresting their division cycle in the G_1 phase and by inducing the synthesis of proteins required for the mating and fusion of **a** and α cells. Similarly, α cells bind and respond to the **a**-factor peptide that is secreted by **a** cells. Genetic and biochemical evidence indicates that pheromone receptors activate a heterotrimeric GTP-binding regulatory protein; upon activation, the Gβγ subunits of this complex stimulate a protein kinase cascade that regulates cell division and the expression of pheromone-responsive genes. As for other G-protein-coupled receptors, the α-factor and **a**-factor receptors span the plasma membrane seven times (12, 16). Both receptors are subject to ligand-induced, as well as constitutive, endocytosis (17, 58). Sequence elements in the C-terminal cytoplasmic domain are essential for both ligand-induced (52, 58) and constitutive (40) endocytosis of the α-factor receptor, whereas the C-terminal domain of the **a**-factor receptor is necessary only for constitutive endocytosis (17). Endocytosed receptors are delivered to the lysosome-like vacuole, where they are degraded. Other yeast plasma membrane proteins are internalized and degraded in response to different regulatory cues; examples include the transporters for uracil (20), inositol (42), maltose (47), and galactose (15), which respond to nutritional changes. The copper transporter, in contrast, is cleaved at its plasma membrane location in response to high extracellular concentrations of copper ions (49).

In this paper, we show that defects in a temperature-sensitive mutant form of the α-factor receptor accelerate the rate at which it is delivered to the vacuole and degraded. These structural defects were recognized both at the plasma membrane and in one or more compartments of the secretory pathway.

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655-0122. Phone: (508) 856-2157. Fax: (508) 856-5920. E-mail: jennessd@ummed.edu.

MATERIALS AND METHODS

Strains and mutant isolation. The *S. cerevisiae* strains used are listed in Table 1. They are congenic to strain 381G (24) except for strains JN54, JN490, LY110,

TABLE 1. Yeast strains used in this study

Strain[a]	Genotype[b]
381G	*MAT***a** *cry1 ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3*(Ts)
3262-14-3	381G *MAT*α *ADE2*$^+$ *ade6 TYR1*$^+$ *sup4-87*
DJ120-6	381G *bar1-1 leu2 TYR1*$^+$
DJ147-1-1	381G *MAT*α *ura3*
DJ147-1-2	381G *leu2 ura3 TYR1*$^+$
DJ147-1-2-pep4Δ	381G *leu2 ura3 TYR1*$^+$ *pep4::URA3*
DJ147-2-2	381G *leu2 TYR1*$^+$
DJ178-2-2	381G *bar1-1 leu2 TYR1*$^+$ *ste2-3*
DJ213-6-3	381G *ste2-10::LEU2 leu2 ura3*
DJ215-7-1	381G *leu2 ste2-3 TYR1*$^+$
DJ240-4-1	381G *ste2-10::LEU2 leu2 ura3 TYR1*$^+$
DJ256-2-1	381G *leu2 ura3 TYR1*$^+$ *ste2-3*
DJ256-2-1-pep4Δ	381G *leu2 ura3 TYR1*$^+$ *ste2-3 pep4::URA3*
DJ256-3-1	381G *ste2-3 ura3 leu2*
DJ256-7-4	381G *ste2-3 ura3*
DJ257-4-2	381G *ste2-6 ura3 leu2*
DJ283-9-2	381G *ste2-3 ura3 can1*(Ts)
JN54	*MAT***a** *his3-11,15 leu2-3,112 ura3-52 trp1*Δ*1 lys2*
JN490	JN54 *sec1*(Ts)
LY110	JN54 *ste2-3*
LY111	JN54 *sec1*(Ts) *ste2-3*

[a] All strains are congenic to strain 381G (24), except for strains JN54, JN490, LY110, and LY111. The designation 381G indicates that the genotype is the same as strain 381G except for the markers shown.

[b] The *bar1-1* mutation (64) inhibits α-factor degradation in **a** cell cultures. The temperature-sensitive *SUP4-3* mutation suppresses amber mutations *his4-580* and *trp1* at 22°C but not at 34°C (24). The *sup4-87* mutation is a nonsuppressing derivative of *SUP4-3* (33). *ste2-10::LEU2* is a deletion of the *STE2* gene.

and LY111. Yeast strains were constructed by using standard genetic crosses, except for strains DJ147-1-2-pep4Δ, DJ256-2-1-pep4Δ, LY110, and LY111, which were constructed by single-step gene replacement (55). Strains DJ147-1-2 and DJ256-2-1 were transformed with the *Xho*I-*Eco*RI fragment from plasmid pTS15 (54) carrying *pep4::URA3*. The presence of the *pep4::URA3* allele was confirmed by PCR analysis. Strains LY110 and LY111 were constructed in two steps. Strains JN54 and JN490 (from John Nelson, University of Wisconsin) were transformed with the 3.3-kb *Bam*HI fragment from plasmid pKSU (from James Konopka, State University of New York, Stony Brook, N.Y.) containing *ste2*Δ*::URA3*. The Ura$^+$ transformants were then transformed with the 4.3-kb *Bam*HI fragment containing the *ste2-3* allele from plasmid pDJ226 to generate strains LY110 and LY111, respectively.

Plasmids. Plasmid pJBK008 (40) is a yeast centromere plasmid containing the *URA3* and *STE2* genes. Plasmid pJBK023 (40) is pJBK008 containing an *Eco*RI linker insertion at codon 326 in the *STE2* gene; the resulting *ste2-T326* allele encodes a truncated α-factor receptor. Plasmid pDJ226 is pJBK008 containing the *ste2-3* mutation. It was constructed by using the plasmid gap repair method (56): plasmid pJBK008 was digested with *Hin*dIII to remove the receptor coding sequence and used to transform strain DJ256-3-1 to the Ura3$^+$ phenotype. The structure of the resulting plasmid was confirmed by using restriction analysis and complementation of a *ste2*Δ strain at 22°C. Plasmid pDJ227 is pJBK008 containing both the *ste2-3* and *ste2-T326* mutations. It was constructed by subcloning the *Hin*dIII-*Pst*I fragment from pDJ226 (containing *ste2-3*) into the corresponding sites of pDJ138 to yield plasmid pDJ148; the *Cla*I-*Pst*I fragment from pJBK023 (containing *ste2-T326*) was then subcloned into the corresponding sites of pDJ148 to create plasmid pDJ224; and finally the *Hpa*I-*Eco*RI fragment from pDJ224 (containing *ste2-3* and *ste2-T326*) was subcloned into pJBK023 to yield plasmid pDJ227. Plasmid pDJ138 is a 1.7-kb *Nsi*I-*Bcl*I fragment containing the *STE2* gene cloned into a yeast integrating plasmid containing the *URA3* gene.

Culture media. YM-1 is a rich liquid medium (23). The minimal medium is yeast nitrogen base (without amino acids) (Difco Laboratories) supplemented with ammonium sulfate (1 mg/ml) as the nitrogen source and glucose (2%) as the carbon source. C medium is minimal medium supplemented with adenine, uracil, arginine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine. The amino acids were used at 40 μg/ml, and uracil and adenine were used at 20 μg/ml. −Ura medium was C medium lacking uracil. −Ura+CAA medium was supplemented with 0.1% Casamino Acids (Difco). Solid media contained 2% agar. Liquid media were buffered to pH 5.8 with succinic acid (10 g/liter) and NaOH.

Antisera. Rabbit polyclonal antisera used in immunoblotting procedures were specific for the N-terminal or the C-terminal portion of the α-factor receptor (40); an integral membrane subunit of the vacuolar ATPase, Vph1p (45); a marker of the endoplasmic reticulum, Kar2p (57); or the Golgi GDPase, Gda1p (1). Mouse monoclonal antibodies C56 (2) were specific for the plasma membrane ATPase, Pma1p (58). The anti-α-factor receptor antibodies were affinity purified by standard methods (51).

Sequencing analysis. As with plasmid pDJ226, the plasmid gap repair method (56) was used to clone the *STE2*$^+$ and *ste2-6* alleles from strains DJ147-1-1 and DJ257-4-2, respectively. The 1.3-kb *Hin*dIII-*Pst*I fragments from these plasmids and from plasmid pDJ226 were cloned into the *Hin*dIII and *Pst*I sites of plasmid pDJ138. The *ste2-3* and *ste2-6* derivatives of plasmid pDJ138 (plasmids pDJ148 and pDJ164, respectively) resulted in a temperature-sensitive mating phenotype when integrated at the *URA3* locus of the *ste2*Δ strain DJ213-6-3, whereas the *STE2*$^+$ derivative (plasmid pDJ152) led to fertility at 34°C. The *Hin*dIII-*Dra*I fragment and the overlapping *Hpa*I-*Aat*II fragment from pDJ148 (*ste2-3*) cloned into the corresponding location of plasmid pDJ138 (*STE*$^+$) resulted in a temperature-sensitive mating phenotype when integrated into strain DJ213-6-3. In contrast, the overlapping *Aat*II-*Pst*I and *Cla*I-*Eco*RI fragments (representing the remaining portion of *ste2-3*) rescued the sterility phenotype of strain DJ213-6-3 when they were cloned into the corresponding sites of pDJ138 and integrated at the *URA3* locus. We conclude that the mutational lesion in the *ste2-3* allele is between the *Hpa*I and *Dra*I sites of the gene. This assignment was confirmed by subcloning the 262-bp *Hpa*I-*Dra*I fragment from pDJ138 (*STE*$^+$) into the corresponding sites in pDJ148 (*ste2-3*); this plasmid rescued the sterility of strain DJ213-6-3 when integrated at *URA3*. Clones containing the *Hin*dIII-*Dra*I and *Hpa*I-*Aat*II fragments from pDJ164 indicated that the mutational lesion in the *ste2-6* allele was also between the *Hpa*I and *Dra*I sites. The *Hpa*I-*Dra*I fragments from pDJ148, pDJ164, and pDJ152 were subcloned into the *Sma*I site of plasmid pUC18, and the DNA sequence of the insert was determined. Both the *ste2-3* and *ste2-6* alleles were found to contain the sequence ACC at codon 52, whereas the (*STE2*$^+$) control contained GCC, consistent with the published sequence of the wild-type allele (10, 48).

Immunoblotting methods and quantitation. Protein samples were diluted 1:3 with sample buffer (1 g of urea dissolved in 1 ml of 17.5 mM Tris-HCl [pH 6.8], 1.75% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol, bromophenol blue) and heated to 37°C for 10 min. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (41) and transferred (67) to an Immobilon membrane (Millipore Corp., Bedford, Mass.). Membranes were blocked with 20 mM Tris-Cl (pH 7.5)–0.5 M NaCl–0.05% Tween 20 containing 5% nonfat dried milk; they were probed with primary antiserum and secondary antibodies diluted in blocking buffer. Secondary antibodies were either goat anti-rabbit or goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). Conjugates were visualized with chemiluminescence reagents (Amersham Life Science, Arlington Heights, Ill.). The resulting signal was quantified with a model SLR-1D/2D Zeineh soft laser-scanning densitometer. The linearity of the detection assay was evaluated by analyzing dilutions of the most highly concentrated sample.

Renografin density gradients. Membranes were prepared and resolved on 22 to 38% Renografin density gradients as described previously (58), except that the cells were cultured at 34°C in YM-1 medium (strains DJ120-6 and DJ178-2-2) or −Ura+CAA medium (strain DJ240-4-1 containing plasmid pJBK008, pJBK023, pDJ226, or pDJ227) and did not receive cycloheximide prior to harvest. Except as noted, samples of the fractions were diluted with sample buffer and loaded directly on polyacrylamide gels for SDS-electrophoretic analysis and specific protein markers were assayed by immunoblotting methods. The exceptions were for the samples that were to be assayed for Vph1p and Kar2p. These samples were first diluted with 2 volumes of membrane buffer (50 mM Tris [pH 7.5], 1 mM EDTA), and the membranes were collected in a Beckman Airfuge (20 min).

Protein turnover assays. Turnover of α-factor receptor protein was monitored under two different sets of experimental conditions for strains DJ147-2-2, DJ147-1-2-pep4Δ, DJ215-7-1, and DJ256-2-1-pep4Δ. Under one condition, the cells were cultured at 34°C overnight in YM-1 medium to a final concentration between 5×10^6 and 2×10^7 cells/ml, diluted to 5×10^6 cells/ml, and then cultured further at 34°C in the presence of 10 μg of cycloheximide per ml. Under the other experimental condition, cells were cultured at 22°C overnight in YM-1 medium to 10^7 cells/ml, diluted with an equal volume of medium at 46°C, and then cultured further at 34°C in the presence of 10 μg of cycloheximide per ml. At each time point, 25-ml samples were removed to tubes containing 10 g of ice, 350 μmol of NaN_3, and 350 μmol of KF. The cells were collected by centrifugation; rinsed in 1 ml of membrane buffer containing 10 mM NaN_3; suspended in 0.2 ml of membrane buffer containing 10 mM NaN_3, 10 mM KF, and protease inhibitors (100 μg of phenylmethylsulfonyl fluoride per ml and 2 μg of pepstatin A per ml); and then lysed by mechanical disruption with glass beads. The lysate was removed and combined with a 0.2-ml rinse of the glass beads. Unbroken cells were removed by centrifugation for 5 min at $330 \times g$ in a 0.65-ml microcentrifuge tube. The protein concentration of the cleared lysate was determined by the bicinchoninic acid method (Pierce, Rockford, Ill.) and adjusted to 1 mg/ml (*ste2-3* strains) or 0.5 mg/ml (*STE2*$^+$ strains) with sample buffer. Samples were heated to 37°C for 10 min, and the proteins were resolved by SDS-PAGE. Two- and fourfold dilutions of each zero-time point were analyzed on the same gel. The relative receptor concentrations were determined by immunoblotting methods.

These methods were modified to monitor receptor turnover in strains JN54, JN490, LY110, and LY111. The strains were first cultured at 30°C and then shifted to 37°C. Half of each culture received 10 μg of cycloheximide per ml

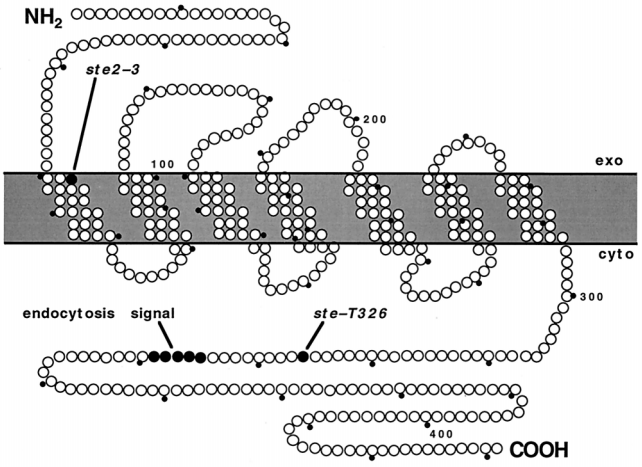

FIG. 1. Topological structure of the α-factor receptor. The structural predictions for the receptor in the plasma membrane are based on its amino acid sequence (10, 48) and on the analysis of chimeric fusion proteins (12). Shown are the positions and orientations of the seven transmembrane segments. Residues 1 to 49 comprise an extracellular N-terminal domain, and residues 296 to 431 comprise an intracellular C-terminal domain. The *ste2-3* mutation was found to result in an Ala→Thr substitution at position 52. The *ste2-T326* mutation leads to a translational frame shift after residue 326 (40) and blocks ligand-mediated endocytosis of the receptor (58). The DAKSS endocytosis signal (residues 335 to 339) is required for α-factor internalization (52) and for the ubiquitination of the receptor that occurs during endocytosis (25).

immediately after the temperature shift; the other half received cycloheximide 30 min after the shift (to permit inactivation of the temperature-sensitive *SEC1* gene product and to allow the depletion of the temperature-sensitive receptors that were present before the shift to 37°C). Samples were withdrawn at intervals, and the amount of receptor protein was assayed as above. The first culture was used as a control for monitoring the loss of receptors that were present before the shift to 37°C. The relative amounts of receptor protein in the second culture were plotted as a function of time after the addition of cycloheximide. An additional control was used to ensure that the 30-min incubation at 37°C was sufficient to block secretory function in the *sec1*(Ts) strains. Strains JN54 and JN490 were transformed with plasmid p21-PGK-SPB, which directs the secretion of *Escherichia coli* β-lactamase (11). Transformants that had been cultured at 30°C were shifted to 37°C for 30 min, and then β-lactamase secretion was assayed by pulse-chase methods (11). Although significant quantities of high-molecular-weight precursor protein were present in both cultures after a 30-min chase period, the *SEC1*$^+$ strain had released 68% of the mature β-lactamase into the culture fluid whereas the *sec1*(Ts) mutant had released only 5% of the mature β-lactamase.

α-Factor binding assays. ^{3}H-labeled α-factor binding assays (34) were carried out as described previously.

RESULTS

Cloning and sequencing the *ste2-3* allele. Haploid **a** cells that contain the *ste2-3* mutation respond to α-factor and mate at 22°C but are unresponsive to α-factor and are sterile at 34°C (24, 33) because of temperature-sensitive defects in the α-factor receptor (31, 33). When grown at 22°C, the *ste2-3* mutant cells bind α-factor at roughly half of the wild-type level (10^4 sites per cell) whereas the mutant cells grown at 34°C contain fewer than 100 binding sites (32, 33, 44). To determine the amino acid substitution responsible for the mutant phenotype, we transferred the *ste2-3* allele from the chromosomal locus to the plasmid shuttle vector pJBK008 by using the plasmid gap repair method (56). Hybrid *STE2* genes that contained various segments of the *ste2-3* mutant allele were then tested for receptor function (see Materials and Methods). The 262-bp *Hpa*I-*Dra*I fragment from the *ste2-3* gene was sufficient to convey the temperature-sensitive mating phenotype, whereas the hybrid constructs that contained the other segments of the mutant gene did not result in a mutant phenotype. The nucleotide sequence of this segment indicated threonine at codon position 52, whereas the published sequence of the *STE2* gene (10, 48) indicates alanine at this position. When the wild-type *STE2*$^+$ allele from the isogenic parent strain was cloned and sequenced, it was found to encode alanine at this position. The independent *ste2-6* allele (24) was also found to contain a threonine substitution at position 52; hence, it is apparently identical to the *ste2-3* allele. This residue is located at the beginning of the first transmembrane segment of the receptor (Fig. 1).

Structure and localization of the mutant receptor protein at the restrictive temperature. We considered three explanations for the failure of *ste2-3* cells to accumulate cell surface α-factor binding sites when cultured at 34°C. Synthesis of the mutant receptors may be blocked; the mutant receptors may fail to accumulate at the cell surface; or they may accumulate at the cell surface in a form that fails to bind α-factor. To distinguish among these possibilities, we examined the abundance, structure, and subcellular location of the mutant receptor protein. In wild-type cells, two major glycosylated forms of the receptor (p53 and p48) are produced that can be converted to the unglycosylated form (p43) by using endoglycosidase H treatment (9, 40). Essentially all of the wild-type receptor protein (Ste2p) copurifies with plasma membranes (58) and is sensitive to cleavage when whole cells are treated with chymotrypsin (58). Figure 2 depicts the results of immunoblot analysis of wild-type and *ste2-3* mutant cells that had been cultured at 34°C. The *ste2-3* mutant produced receptors (Ste2-3p) that comigrated with the two major glycosylated species, p53 and p48; thus, the Ste2-3p apparently enters the secretory pathway. The steady-state level of Ste2-3p, however, was reduced sixfold compared with Ste2p (compare lanes 1 and 2 with lanes 5 and 6). The reduced accumulation of Ste2-3p apparently resulted from degradation of the mutant receptors in the vacuole, since Ste2-3p and Ste2p accumulated to equal levels in the *pep4* mutant (compare lanes 3 and 4 with lanes 7 and 8)—*pep4* mutant cells are deficient for the major proteolytic activities of the vacuole (35).

Two observations pertaining to the electrophoretic mobility of Ste2-3p in the *pep4* mutant are of interest. First, higher-molecular-weight forms of the mutant receptor accumulated

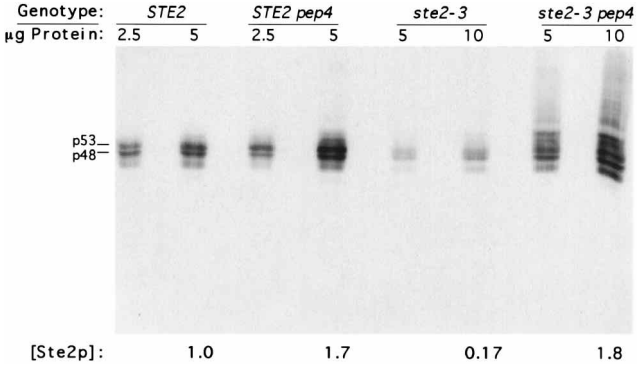

FIG. 2. Analysis of receptor protein in the *ste2-3* mutant. Cleared-cell lysates were resolved by SDS-PAGE, and the α-factor receptor was detected by anti-C-terminal Ste2p and immunoblotting methods. The relevant genotypes and the total amount of protein analyzed (micrograms of protein) are indicated at the top of the figure. The relative concentration of receptor protein in the cell was estimated from densitometric analysis of the immunoblot (scanner units divided by total protein analyzed) and normalized to the wild-type value. p48 and p53 are the two major glycosylated receptor species. The strains used are DJ147-2-2 (*STE2*), DJ147-1-2-pep4Δ (*STE2 pep4*), DJ215-7-1 (*ste2-3*), and DJ256-2-1-pep4Δ (*ste2-3 pep4*).

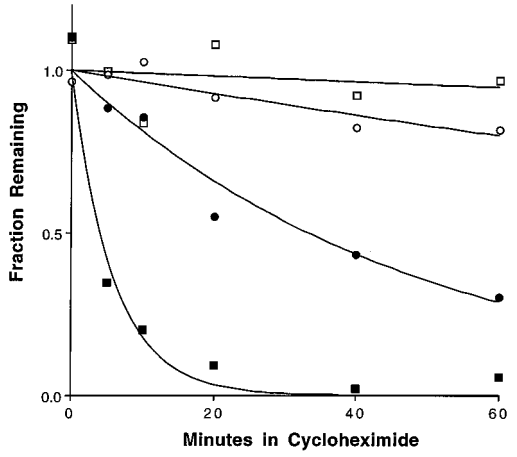

FIG. 3. Turnover of receptor protein at 34°C. Cultures that had been growing exponentially at 34°C were treated with cycloheximide at time zero. Samples were removed at the time points indicated, and the receptor protein concentration was assayed as in Fig. 2. Each point represents the average of four measurements: two receptor protein assays for each of two independent cultures. Results were fitted to a first-order decay curve and normalized to the zero time value. Symbols: ●, *STE2* (strain DJ147-2-2); ○, *STE2 pep4* (DJ147-1-2-pep4Δ); ■, *ste2-3* (DJ215-7-1); □, *ste2-3 pep4* (DJ256-2-1-pep4Δ).

when degradation was blocked in the *pep4* mutant (lanes 7 and 8). The spacing of these electrophoretic species is consistent with multiubiquitination (14, 19). Although we have not directly demonstrated that these receptors are conjugated to ubiquitin, both the α-factor and the **a**-factor receptors are known to be ubiquitinated during ligand-induced endocytosis (25, 53). Second, we detected no degradation intermediates of Ste2-3p when immunoblots were probed with antiserum that recognizes the N-terminal domain of the receptor (exposed to the lumen of the vacuole) (not shown) or with antiserum that recognizes the C-terminal domain (exposed to the cytosol) (Fig. 2). This method is known to detect differences in the rates of cytosolic and extracellular cleavages since treatment of wild-type cells with chymotrypsin results in fragments of intermediate length that are readily detected with antiserum specific for the C-terminal cytosolic domain (58). Thus, it appears that both faces of Ste2-3p are degraded in concert and that this degradation requires the action of vacuolar proteases.

To test whether the reduced cellular accumulation of the mutant receptors at 34°C was due to an increased rate of degradation, we compared the turnover rates of Ste2-3p and Ste2p. Cycloheximide was used to block new receptor synthesis in cells that had been cultured continuously at 34°C, and the relative accumulation of Ste2-3p and Ste2p was monitored over a 60-min time course (Fig. 3). Both the mutant and the wild-type receptors showed first-order decay rates with half-lives of 4 and 33 min, respectively. This roughly eightfold increase in turnover rate easily accounts for the sixfold decrease in the steady-state level of Ste2-3p (Fig. 2); thus, the reduced accumulation of Ste2-3p is apparently due to an increased rate of receptor degradation rather than a decreased rate of synthesis. Degradation of both Ste2-3p and Ste2p was completely blocked in the *pep4* mutant.

The subcellular location of the mutant receptors was determined by Renografin gradient centrifugation. We previously found that this technique efficiently resolves plasma membranes from the membranes of the ER, Golgi complex, and vacuole (27, 58). The membrane fractionation pattern obtained with the *ste2-3* mutant grown at 34°C (Fig. 4B) was compared with the pattern obtained with the *STE2*$^+$ control cells (Fig. 4A). The wild-type Ste2p accumulated at the plasma membrane (Fig. 4A), whereas the mutant Ste2-3p was restricted to internal compartments (Fig. 4B). Although plasma membranes were resolved from the intracellular membranes, the membranes from the various internal compartments were not resolved from each other. Other fractionation techniques requiring spheroplast formation proved to be unsatisfactory because significant quantities of Ste2-3p were degraded during cell wall digestion. When the *ste2-3* mutant was cultured at 22°C, essentially all the Ste2-3p was recovered in the plasma membrane fractions (data not shown). In contrast, the Ste2-3p from *ste2-3 pep4* cells that had been cultured at 34°C was restricted to the internal membrane fractions (data not shown). Furthermore, green fluorescent protein that had been coupled to the C terminus of Ste2-3p was found to accumulate in the vacuole (44). These results, taken together with the *PEP4*-dependent turnover of the receptors (Fig. 3), indicate that essentially all Ste2-3p synthesized at 34°C enters the secretory pathway and is delivered to the vacuole, and that significant

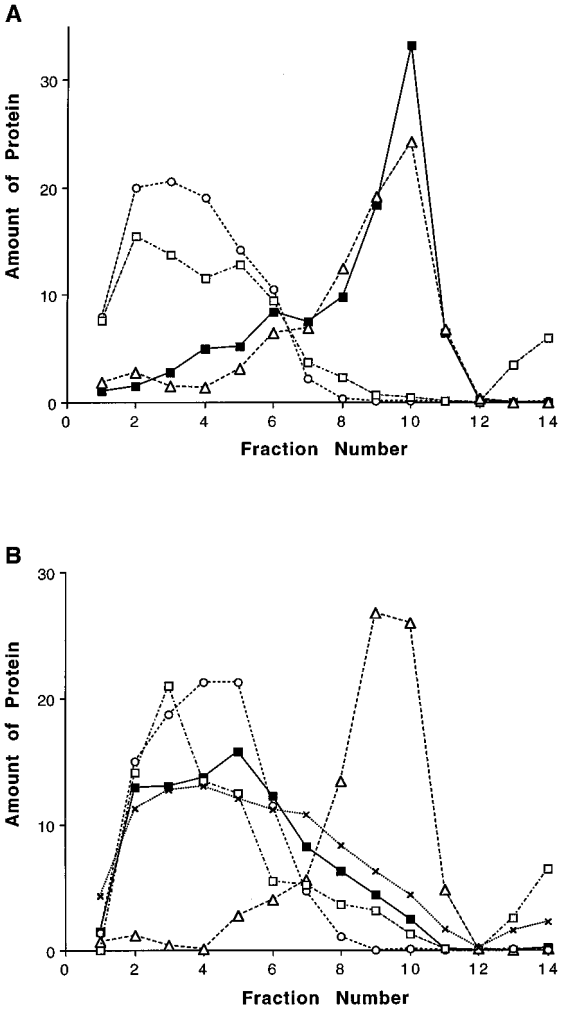

FIG. 4. Renografin gradient fractionation of membranes from the wild-type and *ste2-3* strains grown at 34°C. Membranes from crude lysates were resolved on 22 to 38% Renografin gradients. Fractions were assayed for α-factor receptor (■); plasma membrane ATPase, Pma1p (△); vacuolar ATPase subunit, Vph1p (○); the Golgi membrane marker, Gda1p (X); and the ER marker, Kar2p (□). (A) Wild-type strain DJ120-6. (B) *ste2-3* mutant strain DJ178-2-2.

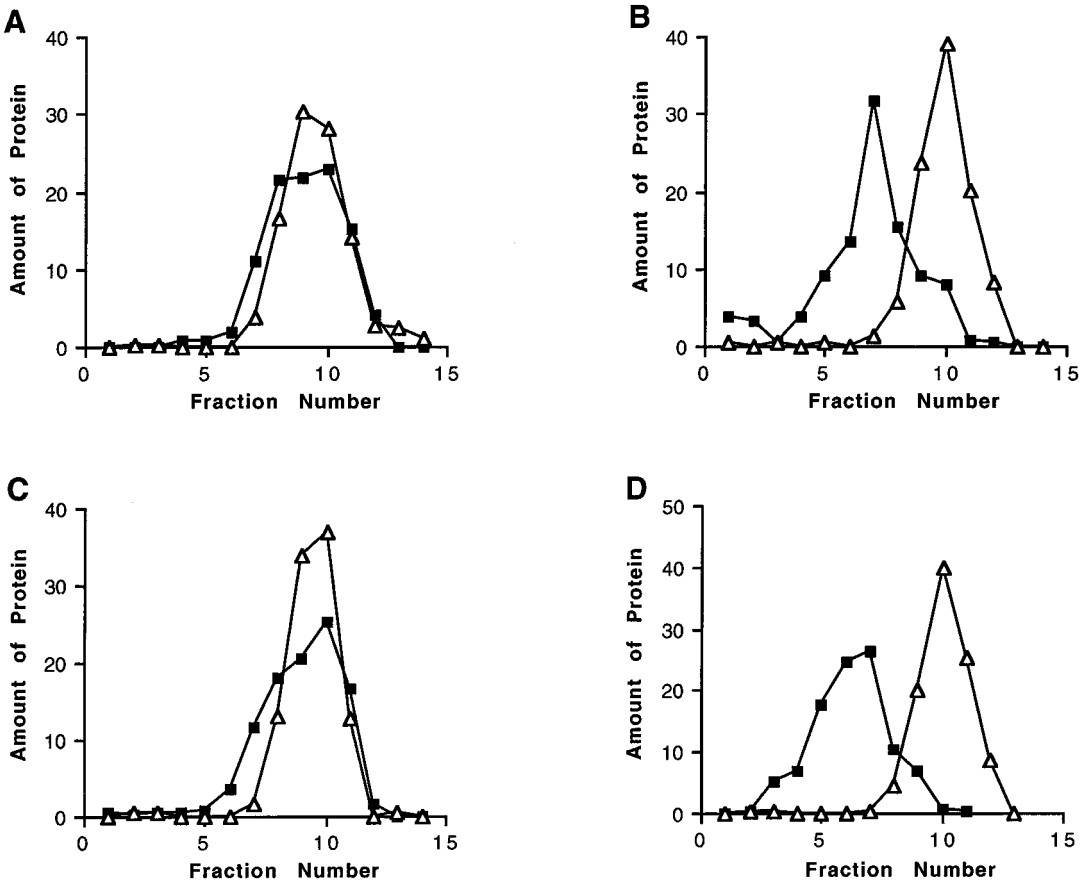

FIG. 5. The C-terminal domain of the receptor is unnecessary for mislocalization of mutant receptors at 34°C. Renografin gradient analysis was performed on *ste2*Δ strain DJ240-4-1 that had been transformed with plasmids containing wild-type or mutant forms of the *STE2* gene. Fractions were assayed for α-factor receptor protein (■) and plasma membrane ATPase, Pma1p (△). Cells were cultured at 34°C in −Ura+CAA medium. (A) Plasmid pJBK008 containing *STE2*$^+$. (B) Plasmid pJBK226 containing the *ste2-3* mutant. (C) Plasmid pDJ023 containing *ste2-T326*. (D) Plasmid pDJ227 containing the *ste2-3,T326* double mutant.

quantities of Ste2-3p do not accumulate at the plasma membrane.

The C-terminal cytoplasmic domain is not required for elimination. The C-terminal cytoplasmic domain of the α-factor receptor is required for both ligand-induced and constitutive endocytosis (40, 52, 58). Truncated receptors encoded by the *ste2-T326* allele (Fig. 1) are defective for both of these endocytic activities due to a translational frameshift at codon 326 in the C-terminal domain (40, 58). We wished to determine whether the endocytic signals missing from this mutant play an essential role in elimination of Ste2-3p. For example, it could be argued that the defective receptors synthesized at 34°C are first delivered to the plasma membrane and that they fail to accumulate on the cell surface because they are endocytosed at a very high rate. To test this hypothesis, we constructed the *ste2-3,T326* allele that contains both the *ste2-3* and the *ste2-T326* mutational lesions. If the endocytic signal is necessary for directing defective receptors to the vacuole, the receptors from the double mutant should accumulate on the plasma membrane when cultured at 34°C. As shown in Fig. 5, the receptor protein encoded by the wild-type and the *ste2-T326* alleles cofractionated with the plasma membrane ATPase (Fig. 5A and C, respectively) whereas the receptors encoded by *ste2-3* and *ste2-3,T326* were associated with the internal membranes (Fig. 5B and D, respectively). Thus, the signals that direct the defective receptors to the vacuole are independent of the endocytic signal in the C-terminal cytoplasmic domain. This observation is inconsistent with the hypothesis that the defective receptors synthesized at 34°C enter the vacuole by the normal endocytic process from the plasma membrane; however, we cannot rule out the unlikely possibility that a novel endocytic signal is created in the mutant receptors.

Elimination of Ste2-3p is not blocked in the *sec1* mutant. The *sec1* mutation blocks the fusion of secretory vesicles with the plasma membrane (36). Hence, when cultures are shifted to the restrictive temperature, *sec1* cells fail to grow and most secreted proteins and integral plasma membrane proteins that were synthesized after the shift become trapped in the secretory vesicles. If elimination of Ste2-3p requires delivery to the plasma membrane, then, in the *sec1* mutant cells, the Ste2-3p that is synthesized at the restrictive temperature should be protected from degradation. Alternatively, if newly synthesized Ste2-3p is diverted to the vacuole before it reaches the plasma membrane, the degradation of Ste2-3p should be unaffected by the *sec1* mutation. In the experiment in Fig. 6, the *sec1* mutant and *SEC1*$^+$ control cells were cultured for 30 min at 37°C to inactivate the mutant *SEC1* gene product and to permit the elimination of the Ste2-3p that had been synthesized during the nonrestrictive conditions, and cycloheximide was then added to block the synthesis of new receptors. Under these conditions, the decay of Ste2-3p was unaffected by the *sec1* mutation, consistent with the hypothesis that newly synthesized Ste2-3p is transported to the vacuole before it reaches the plasma membrane. These results apparently reflected the elim-

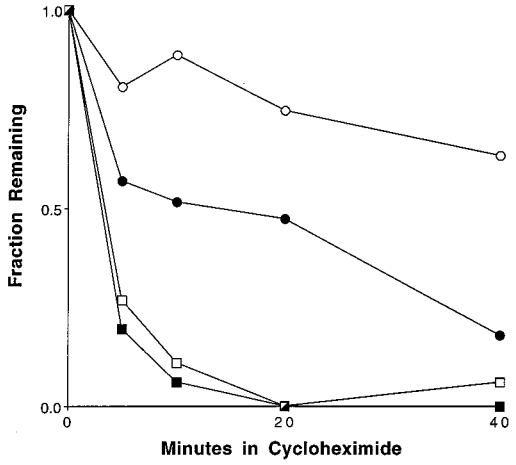

FIG. 6. Effect of mutation *sec1*(Ts) on receptor turnover. Strains LY110 (*ste2-3 SEC1*$^+$) (■), JN54 (*STE2*$^+$ *SEC1*$^+$) (●), LY111 [*ste2-3 sec1*(Ts)] (□), and JN490 [*STE2*$^+$ *sec1*(Ts)] (○) were cultured overnight at 30°C and then shifted to 37°C. After 30 min, the cultures received 10 μg of cycloheximide per ml. At the time points indicated, samples were removed and assayed for receptor protein as in Fig. 2. In control cultures that received cycloheximide immediately after the shift to 37°C, the amount of receptor protein remaining was 68% (strain JN54), 64% (strain JN490), and undetectable (strains LY111 and LY110).

ination of protein that had been synthesized under restrictive conditions, since no Ste2-3p remained after 30 min in the control culture that received cycloheximide immediately after the shift to 37°C (data not shown). In contrast, wild-type receptors decayed more slowly in the *sec1* mutant cells (Fig. 6), suggesting that they must reach the plasma membrane before they can be degraded. The residual decay of wild-type Ste2p in the *sec1* mutant probably reflects the degradation of the receptors that had remained at the cell surface after the 30-min preincubation period, since 64% of the wild-type receptors were present after 30 min in the control culture that had received cycloheximide immediately after the shift to 37°C.

Elimination of mutant receptors pre-assembled at the plasma membrane. We wished to determine whether defective receptors are subject to elimination after they have already been assembled in the plasma membrane. To this end, *ste2-3* mutant cells were first cultured at 22°C (permitting the mutant receptors to accumulate on the cell surface) and then shifted to 34°C (to impose the restrictive conditions). Under these conditions, the α-factor binding sites were eliminated from the surface of the *ste2-3* mutant cells in a time- and energy-dependent manner (Fig. 7); that is, the binding sites disappeared from the *ste2-3* mutant but not from the wild-type control cells, and this loss of binding activity was blocked when the culture received metabolic inhibitors (NaN_3 and KF) at the time of the temperature shift. Although the mutant receptors retained binding activity at 34°C when energy metabolism was absent, they contained structural defects that led to irreversible inactivation at higher temperatures (Fig. 8). In the experiment in Fig. 8, cultures that had been grown continuously at 22°C were shifted to higher temperatures (ranging from 34 to 52°C) and received metabolic inhibitors at the time of the shift. The α-factor binding activity remained relatively stable after the cells had been exposed to temperatures ranging from 34 to 46°C; however, as previously noted (31), the binding sites were inactivated quantitatively at temperatures higher than 49°C. Therefore, subtle structural defects at 34°C (i.e., defects compatible with the retention of ligand binding) are apparently recognized by an energy-dependent process that leads to elimination of the α-factor binding sites at the cell surface.

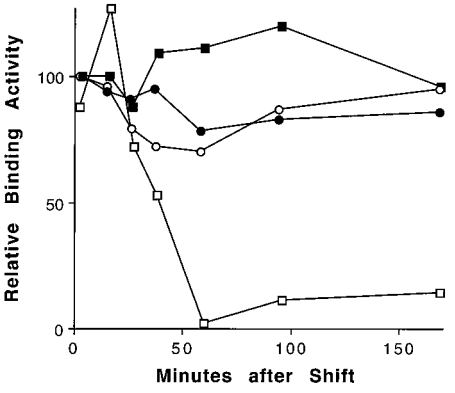

FIG. 7. Metabolic poisons NaN_3 and KF block receptor elimination at 34°C. Strains DJ178-2-2 (*bar1 ste2-3*) (□ and ■) and DJ120-6 (*bar1 STE2*$^+$) (○ and ●) were cultured overnight at 22°C. At time zero, they were shifted to 34°C in the absence (□, ○) or presence (■ and ●) of 10 mM NaN_3 and 10 mM KF. At the time points indicated, samples were removed and assayed for α-factor binding capacity.

Two mechanisms are consistent with specific elimination of the mutant receptors from the plasma membrane. On one hand, at 34°C the mutant receptors may be removed more rapidly than wild-type receptors from the plasma membrane. On the other hand, delivery of mutant receptors to the cell surface may be blocked at 34°C, and the decay of the cell surface binding sites may simply reflect the normal rate of receptor turnover at the cell surface. These two models are not mutually exclusive. To test the first model, we examined the turnover of both receptor protein and α-factor binding sites in the absence of new protein synthesis. Mutant and control cells that had been cultured continuously at 22°C were shifted to 34°C and received cycloheximide to block new receptor synthesis. The mutant receptor binding sites were eliminated at more than twice the rate of the wild-type binding sites (Fig. 9A); the half-lives for the mutant and wild-type binding sites were 32 and 71 min, respectively. This difference between wild-type and mutant receptors was observed in four independent experiments. As depicted in Fig. 9B, the turnover of receptor protein was also influenced by the *ste2-3* mutation;

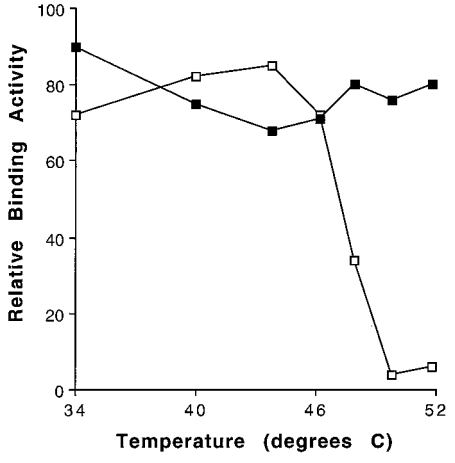

FIG. 8. Thermal stability of wild-type and *ste2-3* mutant receptors. The *ste2-3* mutant strain DJ178-2-2 (□) and the wild-type strain DJ120-6 (■) were cultured at 22°C and then treated with the metabolic poisons NaN_3 and KF. Samples were treated for 30 min at the temperature indicated and then assayed for α-factor binding activity at 25°C.

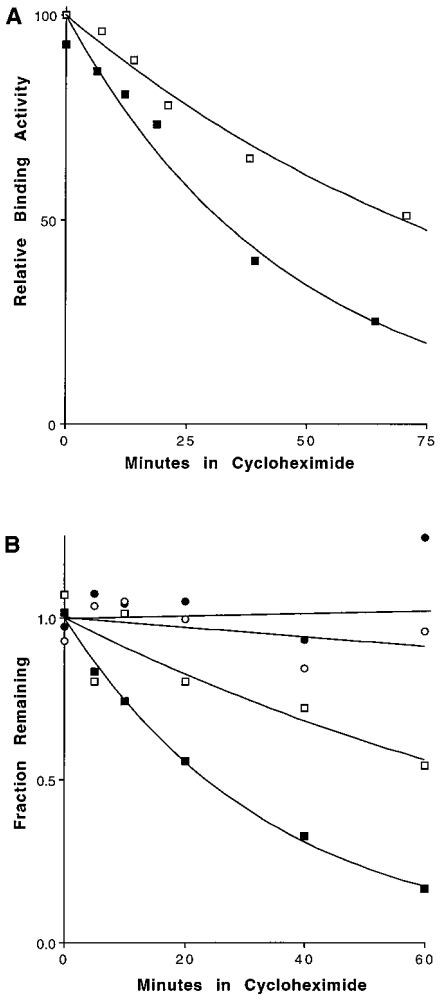

FIG. 9. Elimination of cell surface receptors at 34°C. (A) Elimination of α-factor binding sites. Strains DJ178-2-2 (*bar1 ste2-3*) (■) and DJ120-6 (*bar1 STE2*$^+$) (□) were cultured overnight at 22°C. At time zero, they were shifted to 34°C and received 10 μg of cycloheximide per ml. At the time points indicated, samples were removed and assayed for α-factor binding capacity. Results were fitted to a first-order decay curve and normalized to the zero time value. (B) Elimination of receptor protein. Strains DJ215-7-1 (*ste2-3*) (■), DJ147-2-2 (*STE2*$^+$) (□), DJ256-2-1-pep4Δ (*ste2-3 pep4*) (●), and DJ147-1-2-pep4Δ (*STE2*$^+$ *pep4*) (○) were cultured overnight at 22°C. At time zero, they were shifted to 34°C and received 10 μg of cycloheximide per ml. At the time points indicated, samples were removed and assayed for receptor protein as in Fig. 2. Each point represents the average of four measurements: two receptor protein assays for each of two independent cultures. Results were fitted to a first-order decay curve and normalized to the zero time value.

half-lives for Ste2-3p and Ste2p were 24 and 72 min, respectively. Receptor degradation was dependent on vacuolar proteases, since no degradation of Ste2-3p or Ste2p was detected in the *pep4* mutant (Fig. 9B). The rate-limiting step in the degradation of the cell surface protein appears to be exit from the plasma membrane rather than proteolysis in the vacuole, because the cell surface α-factor binding sites disappear at approximately the same rate as Ste2-3p is degraded (compare Fig. 9A and B) and because, at intermediate time points, all the detectable receptor was in the plasma membrane fractions when the membranes were resolved on Renografin gradients (data not shown). We conclude that upon the temperature shift, the bulk of Ste2-3p at the cell surface was eliminated because of an accelerated removal from the plasma membrane. However, this 2- to 3-fold increase in the receptor turnover rates cannot account for the roughly 100-fold difference in the steady-state levels of binding sites when the *ste2-3* mutant and wild-type control cells were cultured continuously at 34°C. If wild-type and mutant receptors were delivered to the plasma membrane at the same rate, a two- to threefold difference in the half-life of cell-surface receptors would cause only a two- to threefold difference in the steady-state accumulation at 34°C. Therefore, the *ste2-3* mutation apparently affects both the delivery of receptors to and their removal from the cell surface.

DISCUSSION

To preserve the functional integrity of the plasma membrane, cells require a means of recognizing and eliminating structural defects in integral plasma membrane proteins. In this report, we provide evidence that yeast cells contain a system that permits them to eliminate defective α-factor pheromone receptors. At least three intracellular processes appeared to operate on these defective receptors. First, the structural defect was recognized both when the receptors were preassembled at the plasma membrane and when they were in transit to the cell surface. Second, the defective receptors were delivered to the vacuole by a mechanism that was independent of their normal endocytic signal. Third, both the luminal and cytosolic domains of the receptor were degraded in concert, and degradation required the vacuolar protease activities that are regulated by the *PEP4* gene. A potential role for ubiquitin in the elimination process was implied by the accumulation of high-molecular-weight forms of the defective receptor in the protease-deficient strain. There are numerous examples of yeast plasma membrane proteins that are removed and degraded in response to regulatory cues—i.e., ligand-mediated endocytosis (17, 58), nutrient deprivation (15, 20, 42, 47), and control of ion homeostasis (49). Other plasma membrane proteins are subject to high constitutive rates of degradation (5, 7, 18, 39). We report the first example in which a plasma membrane protein is eliminated from the cell surface because of a structural defect.

At least two mechanisms exist for recognizing and destroying defective proteins that are in transit to the plasma membrane. Some defective proteins are diverted from the secretory pathway to the vacuole (13, 29), whereas others are retained in the ER and degraded by the ER degradation system (26, 46, 63, 68). The *ste2-3* mutant receptors are degraded predominantly in the vacuole, since the *pep4* mutation blocks degradation. Furthermore, when the *ste2-3* mutant cells were cultured continuously at 34°C, the newly synthesized mutant receptors were apparently diverted directly to the vacuole without traversing the plasma membrane. This conclusion is supported by four observations. First, in the membrane fractionation studies, we detected no mutant receptor proteins associated with the plasma membrane fractions. Second, the mutant receptors accumulated in the vacuole even when the normal endocytic signal had been removed. Third, degradation of newly synthesized Ste2-3p was unaffected by the *sec1* mutation. Fourth, the receptors that were synthesized at 34°C were degraded more rapidly (4-min half-life [Fig. 3]) than were the receptors that were preassembled at the plasma membrane before they were subjected to the 34°C conditions (24-min half-life [Fig. 9B]). Temperature-sensitive forms of the yeast [H^+] plasma membrane ATPase (Pma1p) also appear to be diverted directly to the vacuole without traversing the plasma membrane (13). For both the mutant Pma1p (13) and Ste2-3p, the defective membrane proteins may be diverted to the vacuole either because they are recognized by elements of a quality control system or

because they lack structural features that actively promote transit to the plasma membrane. At least for Ste2p, the former possibility appears more likely, since overproduction of another defective membrane protein, arginine permease, appears to compete for the elimination of Ste2-3p, in that the *ste2-3* mutant is partially suppressed (our unpublished results). Recent evidence (29) also suggests that receptors in the Golgi complex recognize misfolded soluble proteins and direct them to the vacuole. When invertase was fused to a mutant form of the λ repressor protein, the hybrid protein was targeted to the vacuole; however, in *vps10* mutant cells, this hybrid was diverted to the cell surface (29). Since the product of the *VPS10* gene serves as the Golgi receptor for targeting carboxypeptidase Y to the vacuole, it may also function as a more general recognition element that diverts misfolded proteins to the vacuole.

Although the majority of the Ste2-3p synthesized at 34°C is degraded in the vacuole, a small portion of the mutant protein may be retained and degraded in the ER. In animal cells, calnexin binds many membrane and soluble proteins of the secretory pathway and results in the retention of defective and incompletely assembled proteins in the ER (6). The degradation of prepro-α-factor in isolated yeast microsomes depends on the product of the calnexin gene, *CNE1* (46); however, the *cne1* mutation apparently does not affect degradation of mutant carboxypeptidase Y by the ER degradation system in vivo (38). Interestingly, the *cne1* mutation causes *ste2-3* mutants to exhibit a small increase in α-factor sensitivity (44, 50); therefore, the binding of calnexin to Ste2-3p may limit its exit from the ER. However, since α-factor sensitivity is stimulated only marginally in the *cne1* mutant and since the *pep4* mutation completely blocks Ste2-3p degradation, the ER degradation system plays at best a minor role in α-factor receptor elimination compared to the role played by vacuolar degradation.

Several recent reports suggest that ubiquitination of yeast plasma membrane proteins triggers their endocytic uptake. Both the α-factor receptor (25) and the **a**-factor receptor (53) are ubiquitinated during ligand-mediated endocytosis. In mutants that are defective for certain ubiquitin-conjugating enzymes (most notably, the *ubc4 ubc5* double mutant), both receptors are ubiquitinated substantially less and are endocytosed more slowly. A *ste2* mutant receptor that is defective for endocytosis does not become ubiquitinated, whereas in the *end4* mutant that blocks the endocytic pathway at an intermediate stage, ubiquitination of both receptors is unimpeded. Together, these results provide a strong correlation between ubiquitination and exit from the plasma membrane. Similar observations show a correlation between ubiquitination and endocytosis of other yeast plasma membrane proteins, including the pheromone transport protein, Ste6p (39), the uracil permease, Fur4p (20), and the multidrug transporter, Pdr5p (18). In mammalian cells, receptors that are subject to ligand-mediated endocytosis and degradation are also ubiquitinated during the process (21, 30, 65). However, from these studies, it is impossible to discern whether ubiquitination causes or is a consequence of endocytosis. The lower endocytic rate in the *ubc4 ubc5* double mutant may easily result from the severe pleiotropic defects of this mutant, since it grows 5 to 10 times more slowly than the wild type, it is induced constitutively for stress responses, and it delivers carboxypeptidase Y to the vacuole 3 times more slowly (25, 62). Moreover, plasma membrane proteins can apparently become ubiquitinated without undergoing endocytosis, because at 34°C Ste2-3p is apparently ubiquitinated and delivered to the vacuole even though it does not traverse the plasma membrane.

Once Ste2-3p reaches the vacuole, the cytosolic and luminal faces of the receptor appear to be degraded in concert, as indicated by the detection of few degradation products of intermediate length when Western blots were probed with antiserum directed either to the C-terminal domain (the cytosolic face) or to the N-terminal domain (the luminal face). Furthermore, no degradation intermediates were detected during the time course experiments (Fig. 2 and 7). This phenomenon is interesting, since the vacuolar proteases affected by the *pep4* mutation are located only in the lumen of the vacuole. Two models might explain this apparent paradox. In the first model, both faces of the receptor may be drawn into the vacuolar lumen before they are degraded, perhaps by autophagy. In yeast cells, autophagy delivers cytoplasmic proteins to the vacuolar lumen during nitrogen starvation (3, 66). Furthermore, fructose-1,6-bisphosphatase is transported from the cytosol to the vacuole upon nutritional upshift (28), and the vacuolar enzyme precursor aminopeptidase I is transported from the cytosol to the vacuole without traversing the secretory pathway (37). Curiously, there is significant overlap among the genes that affect autophagy and cytoplasm-to-vacuole targeting (22, 60). In the second model, degradation of the luminal face of the receptor may cause the opposite face to become more susceptible to degradation by cytosolic proteases (perhaps by ubiquitin-dependent proteolysis). An analogous mechanism has been proposed to explain the concerted degradation of certain mitochondrial membrane proteins (43). We are presently examining a mutant that uncouples the degradation of the two membrane faces of Ste2-3p to distinguish between these two possibilities.

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