Ubiquitination of the Yeast a-Factor Receptor

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Abstract. The **a**-factor receptor (Ste3p) is one of two pheromone receptors in the yeast *Saccharomyces cerevisiae* that enable the cell–cell communication of mating. In this report, we show that this receptor is subject to two distinct covalent modifications—phosphorylation and ubiquitination. Phosphorylation, evident on the unstimulated receptor, increases upon challenge by the receptor's ligand, **a**-factor. We suggest that this phosphorylation likely functions in the adaptive, negative regulation of receptor activity. Removal of phosphorylation by phosphatase treatment uncovered two phosphatase-resistant modifications identified as ubiquitination using a *myc*-epitope–tagged ubiquitin construct. Ste3p undergoes rapid, ligand-independent turnover that depends on vacuolar proteases and also on transport of the receptor from surface to vacuole (i.e., endocytosis) (Davis, N.G., J.L. Horecka, and G.F. Sprague, Jr., 1993 *J. Cell Biol.* 122:53–65). An *end4* mutation, isolated for its defect in the endocytic uptake of α-factor pheromone (Raths, S., J. Rohrer, F. Crausaz, and H. Riezman. 1993. *J. Cell Biol.* 120:55–65), blocks constitutive endocytosis of the **a**-factor receptor, yet fails to block ubiquitination of the receptor. In fact, both phosphorylation and ubiquitination of the surface-bound receptor were found to increase, suggesting that these modifications may occur normally while the receptor is at the cell surface. In a mutant strain constructed to allow for depletion of ubiquitin, the level of receptor ubiquitination was found to be substantially decreased. Correlated with this was an impairment of receptor degradative turnover—receptor half-life that is normally ~20 min at 30°C was increased to ~2 h under these ubiquitin-depletion conditions. Furthermore, surface residency, normally of short duration in wild-type cells (terminated by endocytosis to the vacuole), was found to be prolonged; the majority of the receptor protein remained surface localized fully 2 h after biosynthesis. Thus, the rates of **a**-factor receptor endocytosis and consequent vacuolar turnover depend on the available level of ubiquitin in the cell. In cells mutant for two E2 activities, i.e., *ubc4Δ ubc5Δ* cells, the receptor was found to be substantially less ubiquitinated, and in addition, receptor turnover was slowed, suggesting that Ubc4p and Ubc5p may play a role in the recognition of the receptor protein as substrate for the ubiquitin system. In addition to ligand-independent uptake, the **a**-factor receptor also undergoes a ligand-dependent form of endocytosis (Davis, N.G., J.L. Horecka, and G.F. Sprague, Jr. 1993. *J. Cell. Biol.* 122:53–65). Concurrent with ligand-dependent uptake, we now show that the receptor undergoes ligand-induced ubiquitination, suggesting that receptor ubiquitination may function in the ligand-dependent endocytosis of the **a**-factor receptor as well as in its constitutive endocytosis. To account for these findings, we propose a model wherein the covalent attachment of ubiquitin to surface receptor triggers endocytic uptake.

Two distinct systems effect cellular protein degradation—the lysosomal system and the ubiquitin-dependent proteasomal system. The two degradation mechanisms act upon different sets of substrates, a consequence of their distinct subcellular compartmentalization. While lysosomal proteases are enclosed within the membrane-bound confines of the lysosome, the proteasome is available to digest both cytoplasmic and nuclear proteins.

Lysosomal degradation requires transport of substrate proteins to the lysosome. This may be by endocytosis, a process that retrieves substrates from the extracellular milieu, as well as surface-resident membrane proteins. Under conditions of cellular stress (e.g., starvation), the process of autophagy also can deliver bulk cytoplasmic proteins to the lysosome for degradation (Seglen and Bohley, 1992).

The selection of substrates for proteasomal degradation involves the covalent addition of the protein ubiquitin to substrate proteins (Finley and Chau, 1991; Varshavsky, 1992). Ubiquitin is a small, 76-residue-long protein that is joined in a pseudo-peptide linkage through its COOH-terminal carboxylic acid to the ε-amino group of lysyl side chains in selectively recognized proteins. Ubiquitin modification serves to mark a particular protein as a substrate for proteasomal degradation. Ubiquitin-regulated protein degradation is an essential step in a diverse number of cel-

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lular processes, ranging from cell growth and division, to processes such as DNA repair, transcriptional regulation, antigen presentation, signal transduction, and the cell cycle (Ciechanover, 1994; Hochstrasser, 1995, 1996).

Although the lysosomal and proteasomal degradative systems are generally considered to be functionally distinct, a possible link between these two systems is suggested by the discovery of ubiquitinated cell surface proteins; the MEL-14 lymphocyte homing receptor (Siegelman et al., 1986), the PDGF receptor (Yarden et al., 1986; Mori et al., 1992), the growth hormone receptor (Leung et al., 1987), the T cell receptor (Cenciarelli et al., 1992), and the IgE receptor (Paolini and Kinet, 1993) have been shown to be modified by ubiquitin. In some cases, the level of ubiquitination was found to increase upon ligand binding (Mori et al., 1992; Cenciarelli et al., 1992; Paolini and Kinet, 1993). The function of the ubiquitin modification for these cell surface proteins remains uncertain. It has been variously suggested to play a role in modulating receptor signaling (Paolini and Kinet, 1993), or in receptor endocytosis and turnover (Mori et al., 1992; Cenciarelli et al., 1992).

Both the lysosomal and proteasomal degradative systems have been clearly preserved through evolution to the yeast *Saccharomyces cerevisiae.* The genetics of the yeast ubiquitination pathway has been extensively studied, and recently, mutations that directly impair proteasome function have been isolated as well (Jentsch and Schlenker, 1995; Hochstrasser, 1995). The lysosomal system in yeast is embodied by the functionally equivalent organelle known as the vacuole (Jones, 1991). The vacuole is filled with various hydrolases (proteases, lipases, phosphatase, etc.) and likewise serves as a potential endpoint of the yeast endocytic pathway. Proteins that have been shown to be endocytosed to, and degraded within, the vacuole include the two yeast pheromone receptors (Davis et al., 1993; Schandel and Jenness, 1994), α-factor (the peptide ligand for one of the two pheromone receptors) (Singer and Riezman, 1990), permeases for uracil (Volland et al., 1994) and inositol (Lai et al., 1995), as well as the **a**-factor exporter Ste6p (Kolling and Hollenberg, 1994; Berkower et al., 1994).

Recent work suggests ubiquitin also as a possible modification of yeast plasma membrane proteins. In the endocytosis-defective *end3* and *end4* mutant cell backgrounds, Ste6p was found to be trapped at the cell surface in a partially ubiquitinated state (Kolling and Hollenberg, 1994). Furthermore, in the *ubc4 ubc5* background, mutants partially defective for ubiquitin conjugation to cellular substrate proteins, the rapid turnover of Ste6p was found to be slowed, suggesting a role for ubiqutination in Ste6p endocytosis or turnover (Kolling and Hollenberg, 1994). Focusing on the endocytic uptake of α-factor and its receptor, recent work from Hicke and Riezman (1996) has implicated ubiquitin in this process as well. They find ligand-dependent ubiquitination of the receptor, as well as a substantial impairment of ligand uptake in the *ubc4 ubc5* background. Most significantly in terms of linking ubiquitination to endocytosis, they find that a single lysine to arginine missense mutation in a truncated version of the α-factor receptor simultaneously blocks both receptor ubiquitination and the receptor-mediated uptake of the α-factor ligand.

This report focuses on the **a**-factor receptor (encoded by the gene *STE3*). This is one of the two yeast pheromone receptors (the other being the α-factor receptor) enabling the cell–cell communication that precedes mating of the two haploid cell types, **a** and α (Schultz et al., 1995). Each cell type secretes a unique peptide pheromone (**a**-factor by **a** cells, and α-factor by α cells) and expresses at its surface the receptor for the pheromone secreted by the other cell type. Binding of pheromone to its cognate receptor activates an intracellular signal transduction pathway that leads to the physiological alterations necessary for mating.

These two receptors are members of the seven transmembrane segment (7-TMS)[1] receptor family, a large family that includes the retinal rhodopsins as well as receptors for many neurotransmitters (Strader et al., 1994). These receptors share two characteristics: (*a*) a common signaling mechanism, whereby intracellular signaling is initiated by the action of the liganded receptor on a conserved heterotrimeric G protein; and (*b*) a common overall structural similarity, consisting of a hydrophobic core of seven transmembrane domains followed at the COOH terminus by a relatively large, hydrophilic, cytoplasmic tail—the COOH-terminal domain (CTD). The hydrophobic core of the seven transmembrane domains is thought to mediate the primary receptor functions: ligand binding and G protein coupling. The CTD appears to be largely regulatory (Dohlman et al., 1991).

The pheromone receptors are subject to two modes of endocytosis—constitutive or ligand-indendent uptake, as well as a ligand-stimulated uptake (Jenness and Spatrick, 1986; Chvatchko et al., 1986; Davis et al., 1993; Schandel and Jenness, 1994). In both cases, endocytosis delivers receptor to the vacuole where it is degraded by vacuolar proteases. For the **a**-factor receptor, constitutive endocytosis is particularly rapid, and as a consequence, Ste3p is quite short-lived with a half-life at 30°C of ~20 min (Davis et al., 1993). Constitutive and ligand-dependent uptake may be mechanistically distinct, as suggested by the finding that the processes can be genetically separated. For instance, the *Δ365* receptor mutant, which removes the last 105 amino acid residues of the 185-residue-long CTD, is wholly disabled for constitutive endocytosis, yet still competent for ligand-dependent uptake (Davis et al., 1993).

In this report, we demonstrate that the **a**-factor receptor is subject to two distinct covalent modifications: phosphorylation and ubiquitination. Phosphorylation, by analogy to other 7-TMS receptors (Dohlman et al., 1991), likely functions to negatively regulate receptor activity. In terms of ubiquitination, we see a ligand-independent or constitutive receptor modification, as well as ligand-dependent ubiquitination. In addition, cellular mutants, defective for this ubiquitination, impair the rate of the **a**-factor receptor's constitutive endocytosis and turnover. Based on our findings, a model is proposed wherein ubiquitin addition plays an initiating role, marking surface receptors destined for endocytic uptake.

1. *Abbreviations used in this paper*: CTD, COOH-terminal domain, MM, minimal medium lacking methionine and cysteine; 7-TMS, seven transmembrane segment.

Materials and Methods

Plasmids

YEp96, YEp105, and pES12 carry on a *TRP1*/2 μm yeast vector, *CUP1*-ubiquitin, *CUP1-myc*-tagged-ubiquitin, and the *CUP1* promoter only (no downstream ubiquitin), respectively (Ellison and Hochstrasser, 1991). The BamHI to ClaI *CUP1*-ubiquitin–containing fragment of each was inserted between the same sites in the *URA3/CEN* vector YCp50 to make pND164 (*CUP1*-ubiquitin), pND165 (*CUP1-myc*-ubiquitin), and pND167 (*CUP1* promoter only). Plasmids pUB23, pUB39, and pUB100 were provided by Vincent Chau (Wayne State University School of Medicine, Detroit, MI) and Dan Finley (Harvard Medicine School, Boston, MA) (Finley et al., 1994). pUB23 (*URA3*) carries a ubiquitin-β-galactosidase fusion construct expressed from the *GAL1* promoter. pUB39 (*LYS2*) carries *CUP1*-expressed ubiquitin. pUB100 carries a Ubi1 tail expression cassette (Finley et al., 1989).

Strains

Genotype and source of the yeast strains used in this work are listed in Table I. Isogenic sets of strains were generated by specific gene replacement. Generally, the allele to be inserted is carried on the *URA3* integrating plasmid pRS306 (Sikorski and Hieter, 1989). A unique restriction cut is used to direct integration of the plasmid at the homologous chromosomal site, creating a tandem Ura^+ integrant with wild-type and mutant allele separated by the plasmid vector sequences. Ura^- colonies are isolated using 5-fluoro-orotic acid (Boeke et al., 1984) and screened for their mutant phenotype. Mutant colonies are chosen only from individual integrants that yield a mixture of colonies showing either wild-type or mutant phenotypes, i.e., from bona fide tandem integrants. Such two-step gene replacements were used to convert *STE3* to *ste3Δ::LEU2* or to *GAL1-STE3*, to convert *ste3Δ*::LEU2 to *GAL1-STE3Δ365*, to convert *PEP4* to *pep4Δ::LEU2*, and to convert *pep4Δ::LEU2* to the unmarked deletion *pep4Δ*. To convert *pep4Δ::URA3* to *pep4Δ*, essentially the same strategy was used, except the unmarked *pep4Δ* was carried on the *LEU2* integrating plasmid pRS305 (Sikorski and Hieter, 1989).

Antisera

Rabbit antiserum raised against a TrpE–STE3 fusion protein (Clark et al., 1988) was affinity purified using the fusion protein coupled to CNBr-activated Sepharose 4B (Davis et al., 1993). A second affinity-purified anti-Ste3p antibody was used exclusively for Fig. 2. This rabbit antibody, which was separately raised against the same TrpE–STE3 fusion protein and affinity purified as above, except against a MalE–STE3 fusion protein (containing the same portion of Ste3p), shows less nonspecific cross-reaction on Western blots.

Immunoblots

Preparation of protein extracts for Western blotting, SDS-PAGE, the transfer to nitrocellulose, and the antibody incubations were as described previously (Davis et al., 1993). Visualization of the antibody-reactive proteins was via the ECL system (Amersham Corp., Arlington Heights, IL).

Phosphatase Digestion

Protein extracts prepared as above in sample buffer (SB: 40 mM Tris/Cl, pH 6.8, 5% SDS, 8 M urea, 0.1 mM EDTA, 1% β-mercaptoethanol) were diluted 500-fold into 1 ml of the phosphatase digestion buffer (20 mM Citrate/Na, pH 6.0, 50 mM NaCl, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Digestion was with 0.06 U (unless otherwise indicated) potato acid phosphatase (Boehringer Mannheim Corp., Indianapolis, IN) for 2 h at 25°C. Reactions were terminated and protein was precipitated with the addition of TCA to 10% and BSA (2 μg/ml) as carrier. After 30 min at 0°C, samples were centrifuged for 20 min in the microfuge at 4°C. Pellets were washed with acetone, dessicated, and then dissolved in 20 μl of SB for 5 min at 65°C.

Table I. Yeast Strains

Strain	Genotype	Reference or Source
227	*MAT***a** *lys1*	Herskowitz lab strain
EG123	*MAT***a** *ura 3 leu2 trp1 can1 his4*	Siciliano and Tatchell, 1984
SM1229	isogenic to EG123, except *mfa1::LEU2 mfa2::URA3*	Michaelis and Herskowitz, 1998
SY1744	*MATα STE3 pep4Δ::URA3 ura3 leu2 ade2-1^{oc} ade1 his6 trp1^{am}*	Davis et al., 1993
SY2601	*MATα STE3 pep4Δ*	This work; A
SY2602	*MATα ste3Δ::LEU2 pep4Δ*	This work; A
SY2635	*MATα GAL1-STE3Δ pep4Δ*	This work; A
RH144-3D	*MAT***a** *ura3 leu2 his4 bar1-1*	Raths et al., 1993
RH268-1C	*MAT***a** *end4-1*	Raths et al., 1993; B
NDY334	*MATα STE3*	This work; B
NDY335	*MATα STE3 end4-1*	This work; B
NDY341	*MATα GAL1-STE3*	This work; B
NDY342	*MATα GAL1-STE3 end4-1*	This work; B
NDY348	*MATα GAL1-STE3 pep4Δ::LEU2*	This work; B
NDY351	*MATα GAL1-STE3 end4-1pep4Δ::LEU2*	This work; B
NDY356	*MATα GAL1-STE3 pep4Δ*	This work; B
NDY359	*MATα GAL1-STE3 end4-1pep4Δ*	This work; B
SUB280	MAT**a** *lys2-801 leu2-3, 112 ura3-52 his3-Δ200 trp1-1 ubi1::TRP1 ubi2-Δ2::ura3 ubi3-Δub2 ubi4-Δ2::LEU2* [pUB39] [UB100]	Finley et al., 1994; C
SUB312	*MAT***a** [pUB23] [pUB 100]	Finley et al., 1994; C
NDY365	*MATα STE3* [pUB39] [pUB100]	This work; C
NDY377	*MATα STE3* [pUB23] [pUB100]	This work; C
NDY421	*MATα STE3 pep4Δ* [pUB39] [pUB100]	This work; C
NDY422	*MATα ste3Δ::LEU2 pep4Δ* [pUB39] [pUB 100]	This work; C
NDY426	*MATα STE3 pep4Δ* [pUB23] [pUB100]	This work; C
MHY501	*MATα STE3 his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*	Chen et al., 1993
MHY508	*MATα STE3 ubc4-Δ1::HIS3 ubc5-Δ1::LEU2*	Chen et al., 1993; D
NDY277	*MATα ste3Δ::LEU2*	This work; D
NDY346	*MATα STE3 pep4Δ*	This work; D

Strains designated *A* in the reference column are isogenic to SY1744. Strains designated *B* are isogenic to RH144-3D. Strains designated *C* are isogenic to SUB280. Strains designated *D* are isogenic to MHY501.

Susceptibility to External Proteases

The treatment of intact cells with Pronase (Calbiochem-Novabiochem Corp., La Jolla, CA) and the subsequent extract preparation were described previously (Davis et al., 1993).

Cell Labeling, Extract Preparation, and Immune Precipitation

An aliquot composed of 2–4 × 10^7 cells from a culture growing in minimal medium lacking methionine and cysteine (MM), supplemented with 0.2% yeast extract, was collected by centrifugation, washed with 5 ml of MM lacking yeast extract (prewarmed to 30°C), centrifuged again, and resuspended in MM lacking yeast extract at a density of 3 × 10^7 cells per ml. Cells were then incubated by end-over-end rotation in a 12-ml plastic tube for 20 min at 30°C, pulse-labeled for 10 min with the addition of [^{35}S]methionine (DuPont-New England Nuclear, Wilmington, DE) to 1 mCi/ml, and then chased with subsequent addition of methionine and cysteine to 50 μg/ml and yeast extract to 0.2%. At the indicated chase time points, aliquots corresponding to 0.1 mCi were removed from the labeling mix, and extracts were prepared and immune precipitated using one of two different protocols.

For analysis of turnover under ubiquitin-depletion conditions, cells were collected by centrifugation, resuspended in 20 μl extraction buffer (EB; 40 mM Tris/Cl pH 8.0, 8 M urea, 0.1 mM EDTA, 1% β-mercaptoethanol), transfered to a tube containing a 15-μl volume of glass beads, and vortexed for 15 s. An additional 20 μl of EB supplemented with 5% SDS was added, and cell lysis was completed with an additional 1 min of vortexing. Samples were immediately heated at 100°C for 5 min, and then stored at 0°C for several h until required for immune precipations. Before the immune precipitation, IgG from 20 μl of Ste3p antiserum was preabsorbed to 20 mg protein A–Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) by end-over-end rotation for 1 h at 4°C in 1 ml of immune precipitation buffer (IP: 10 mM Tris/Cl, pH 8.0, 0.1% Triton X-100, 2 mM EDTA). The resultant antibody–Sepharose complexes were washed with 1 ml IP, and then resuspended finally in 1 ml IP. Just before initiating the immune precipitations, labeled extracts were again heated at 100°C for 5 min. Then, extracts were diluted with 0.9 ml IP with 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin, and centrifuged for 2 min at 10,000 rpm. The supernatant was then removed to a new tube containing 50 μl of the antibody–Sepharose complex and allowed to react for 3 h at 4°C. Immune complexes were washed once with 1 ml IP plus 0.1% SDS, and then the precipitated protein was eluted from the antibody–Sepharose by incubation for 5 min at 100°C in 40 μl EB containing 2.5% SDS. After centrifugation at 10,000 rpm for 2 min, eluted protein was again diluted into 0.9 ml IP with 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. A second round of immune precipitation was then performed exactly as described above for the first, except in this case, immune complexes were washed four times with IP plus 0.1% SDS. Immune complexes were eluted by incubation for 5 min at 100°C in 30 μl SB.

For analysis of turnover in the *ubc* mutant backgrounds, cells at the indicated times were removed into 2 vol of ice-cold MM that contained 0.2% yeast extract and 15 mM NaN_3 and KF, and then left at 0°C for 20 min. Cells were then collected with a microfuge spin, resuspended in 40 μl oxalyticase buffer (OB: 1.4 M sorbitol, 25 mM Tris/Cl, pH 7.5, 10 mM NaN_3, 10 mM KF, 2 mM $MgCl_2$, 0.3% β-mercaptoethanol) containing oxalyticase (Enzogenetics, Corvallis, OR) at 600 U/ml, and then converted to spheroplasts by incubating at 30°C for 30 min. Spheroplasts were collected by microfugation, and then lysed with addition of 40 μl SB containing 2.5% SDS and incubation at 100°C for 5 min. Extracts were immune precipitated as described above, except that only a single round of immune precipitation was used. Immune complexes were washed four times with IP plus 0.1% SDS and eluted as described above.

Quantitation

The percentage of the receptor proteins either subject to ubiquitination, subject to digestion to external proteases, or remaining in a pulse-chase turnover at a particular timepoint, was roughly estimated by coelectrophoresis of the experimental samples, with dilutions of the sample corresponding to the receptor in its initial condition: for pulse-chase analysis, this would be receptor at the initial timepoint; for the external proteolysis protocol, this would be the mock-digested receptor; for estimation of fraction of receptor ubiquitinated, this would be the bulk unmodified receptor. Comparison of the undiluted sample in its final state (e.g., ubiquitinated receptor or receptor remaining after proteolysis) to the diluted samples allowed rough quantitation.

Pheromone Treatment

Cell cultures were treated with **a**-factor by addition of 0.1 vol of a 20-fold concentrated cell-free filtrate prepared from a saturated culture of EG123 cells transformed with the **a**-factor overproduction plasmid pKK16 (Kuchler et al., 1989). Mock **a**-factor preparations were obtained from the isogenic *mfa1::LEU2 mfa2::URA3* strain SM1229. Concentration of the pheromone filtrates was by partial lyophilization yielding an ~10-fold concentration, followed by further concentration via ultrafiltration with Centriprep-10 (Amicon Corp., Danvers, MA). Growing cultures were treated with 0.1 vol of the concentrated **a**-factor or mock **a**-factor filtrates.

Results

The a-Factor Receptor Is Phosphorylated and Ubiquitinated

Serine/threonine phosphorylation functions in the adaptive regulation of a number of 7-TMS receptors (Dohlman et al., 1991). This is best documented for the β-adrenergic receptor and for the retinal rhodopsins where the negative regulation of signaling activity after stimulus exposure involves phosphorylation at multiple serine and threonine residues within the receptor's CTD by β-adrenergic receptor kinase, or, in the case of rhodopsin, by the β-adrenergic receptor kinase homolog rhodopsin kinase. In yeast, the α-factor receptor has been shown to undergo serine/threonine phosphorylation at multiple sites within its CTD, which has also been suggested to mediate the negative regulation of pheromone receptor activity (Reneke et al., 1988; Konopka et al., 1988; Zanolari et al., 1992; Chen and Konopka, 1996). Resting cells that have not been exposed to the α-factor ligand show a basal level of phosphorylation of CTD serine and threonines. This level of receptor phosphorylation is increased upon α-factor exposure.

The **a**-factor receptor appears in Western analysis as a disperse cluster of bands centered around a molecular mass of 48 kD. Treatment of cellular protein extracts with potato acid phosphatase resulted in the collapse of this cluster into a single gel band with an apparent molecular mass of 46.5 kD (Fig. 1). Therefore, like the α-factor receptor, the unstimulated **a**-factor receptor also is subject to basal phosphorylation. However, longer exposures of this Western blot revealed that not all of the receptor protein had been shifted by the phosphatase treatment to the 46.5-kD size. Two phosphatase-resistant species at 51 and at 57 kD were observed, which together represent ~10% of the total receptor protein.

A candidate for this phosphatase-resistant modification is ubiquitin. The addition of one or two 76-residue-long ubiquitin moieties might account for the discrete shift to the two phosphatase-resistant modified forms at 51 and 57 kD. To test this, we have made use of the *myc*-epitope–tagged ubiquitin system developed by Ellison and Hochstrasser (1991). *myc*-tagged ubiquitin has been shown to substitute efficiently for wild-type ubiquitin in the transfer to several known substrates for ubiquitination (Hochstrasser et al., 1991; Madura and Varshavsky, 1994). Protein extracts were prepared from cells expressing the normal level of chromosomally-derived ubiquitin (Fig. 2, lane *1*), or from the isogenic cells overproducing either the 76-resi-

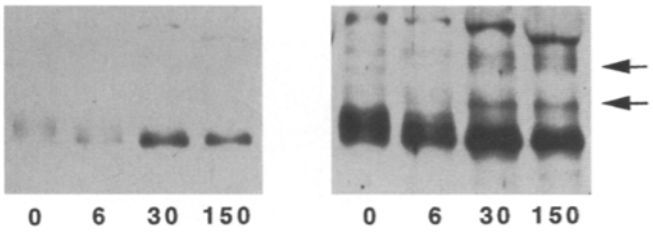

Figure 1. Phosphatase-mediated removal of **a**-factor receptor phosphorylation. Protein extracts prepared from *MATα pep4Δ* cells (SY2601) were treated with increasing concentrations of potato acid phosphatase as described in Materials and Methods. Phosphatase-treated extracts were subjected to SDS-PAGE and Ste3p was visualized by Western analysis using anti-Ste3p antibodies. The milliunits used in each digestion are indicated below. Two exposures of the same experiment are shown. (*Left*) Lighter exposure where the electrophoretic shift accompanying phosphate removal is apparent. As equal aliquots of extract were treated with phosphatase, the light appearance of the cluster of receptor species at 0 and at 6 milliunits of phosphatase likely reflects a dispersion of receptor molecular weights due to heterogeneous phosphorylation. At the highest phosphatase concentration (150 milliunits), some loss of receptor protein is seen, likely a reflection of low level protease contamination of the phosphatase. In the darker exposure (*right*), the positions of the two phosphate-resistant modified receptor forms, most evident in samples treated with 30 and 150 milliunits of phosphatase, are shown (*arrows*). The high molecular weight bands without adjacent arrows are yeast proteins that cross-react with the Ste3p antiserum, as they also appear in samples treated in parallel, derived from a *ste3Δ::LEU2* strain (data not shown).

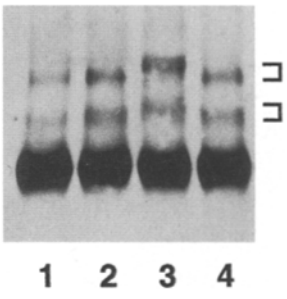

Figure 2. Assessment of **a**-factor receptor ubiquitination. Protein extracts were prepared from *MATα pep4Δ* cells (SY2601), carrying one of three ubiquitin-expression plasmids: the vector control plasmid pND167 that carries only the *CUP1* promoter but no downstream ubiquitin coding sequences (lane *1*); pND164 with *CUP1*-driven wild-type ubiquitin (lanes *2* and *4*); or pND165 with *CUP1*-driven *myc*-tagged ubiquitin (lane *3*). Ubiquitin expression from the *CUP1* promoter was induced in growing cultures with addition of 100 μM $CuSO_4$, 5 h before the collection of cells for extract preparation. Protein extracts were treated with potato acid phosphatase and subjected to SDS-PAGE, and Ste3p was visualized by Western analysis using anti-Ste3p antibodies. The position of the phosphatase-resistant modified receptor species is indicated at right (*brackets*).

due-long wild-type ubiquitin (Fig. 2, lanes *2* and *4*) or the 90-residue-long epitope-tagged ubiquitin (Fig. 2, lane *3*) from the copper-inducible *CUP1* promoter. The two *CUP1*-ubiquitin plasmids result in approximately fivefold overexpression of either wild-type or *myc*-tagged ubiquitin in these cells (data not shown). Because of the slightly enlarged size of the tagged ubiquitin relative to the wild-type form, protein substrate species that receive the *myc*-tagged ubiquitin should show slightly retarded gel mobility relative to the protein modified with the wild-type ubiquitin. As before, protein extracts prepared from these cells were treated with phosphatase, and the electrophoretic mobility of the phosphatase-resistant modified receptors was examined by Western analysis. As seen previously (Fig. 1), two phosphate-resistant, higher molecular weight forms of the **a**-factor receptor were observed in extracts from wild-type cells (Fig. 2, lane *1*) or from cells overexpressing wild-type ubiquitin from the *CUP1*–ubiquitin construct (Fig. 2, lanes *2* and *4*). In extracts prepared from cells overexpressing the *myc*-tagged ubiquitin (Fig. 2, lane *3*), these modified receptor species were shifted, showing slightly retarded gel mobility (Fig. 2, lane *3*; compare with lanes *2* and *4*). We conclude that the phosphatase-resistant receptor modification is, in fact, ubiquitination.

An Endocytosis Blockade Increases Ubiquitination of Surface Receptor

We have examined the effect of the *end4* mutation on receptor ubiquitination. Isolated for its defect in the endocytic uptake of the α-factor ligand (Raths et al., 1993), *end4* also has been demonstrated to block the endocytosis of both Ste6p and the uracil permease (Kolling and Hollenberg, 1994; Berkower et al., 1994; Volland et al., 1994). In Fig. 3, the effect of *end4* on the constitutive endocytosis of the **a**-factor receptor was assessed. The strains in this experiment contain an integrated *GAL1-STE3* allele replacing the wild-type *STE3* locus. Receptor was expressed during 2 h of growth on galactose-containing medium; then, after shutting off further synthesis of the receptor by glucose-mediated repression of the *GAL1* promoter for 45 min, the endpoint localization of the receptor was assessed. The cells used for this experiment were also *pep4Δ*. This mutation blocks the activation of vacuolar zymogens and therefore degradative turnover of the receptor. Thus, in the *pep4Δ* background, receptor delivered to the vacuole (the normal endpoint for Ste3p endocytosis) is stable. To assess localization, the susceptibility of receptor protein in intact yeast cells to added extracellular proteases was measured. This provides a measure of the fraction of receptor proteins localized to the cell surface (Davis et al., 1993). Surface-localized receptor is digested, while internalized receptor is resistant to the external proteases. In the wild-type background (*END4*$^+$), receptor was resistant to the added protease (Fig. 3). This is consistent with endocytotic delivery of preexisting surface receptor to the vacuole during the 45-min period after termination of new receptor protein synthesis by glucose addition. In contrast, in the *end4-1* mutant background, most of the receptor protein was found to be susceptible to the added external protease. This was evident both in the disappearance of full-length receptor and in the appearance of a characteristic proteolytic digestion product at 30 kD, a membrane-protected portion of the receptor corresponding to the seventh transmembrane domain plus the CTD (Fig. 3; Davis et al., 1993). Therefore, as has been shown for other endocytosed surface proteins in yeast, *end4* clearly disrupts the constitutive, ligand-independent endocytosis of the **a**-factor receptor as well.

The *end4-1* allele used in Fig. 3 is temperature sensitive: cell viability is lost at 36°C. α-Factor endocytosis in this background is also temperature sensitive (Raths et al., 1993). However, the effect of this mutation on the constitutive endocytosis of the **a**-factor receptor was found to be

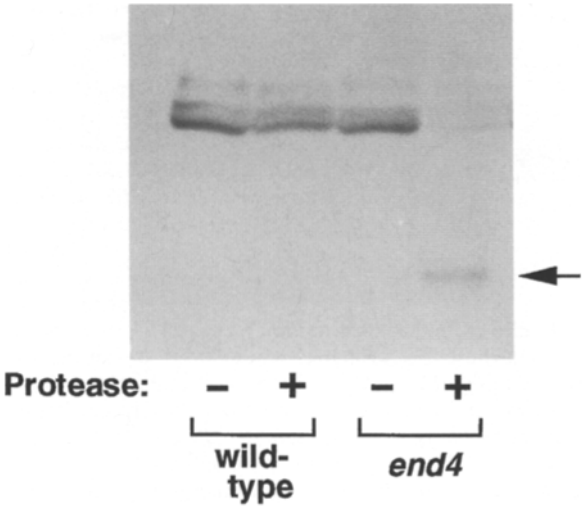

Figure 3. Susceptibility of the **a**-factor receptor in *end4* cells to exogenous protease. Cell cultures from two isogenic *MATα GAL1-STE3 pep4Δ* strains that differ only in being *end4* mutant (NDY359) or wild-type *END4* (NDY356) were grown for 2 h in the presence of 2% galactose, inducing synthesis of the receptor. Receptor synthesis was then shut off with the addition of 3% glucose, and 45 min later, the abundance of receptor at the cell surface in these two backgrounds was assessed by subjecting intact cells to surface proteolysis (+) or to mock protease treatment (−), as described in the Materials and Methods. Subsequent to this, cells were lyzed and extracts were prepared and subjected to SDS-PAGE, followed by Western analysis with the anti-Ste3p antibody. The position of the 30-kD digestion product is indicated at right (*arrow*).

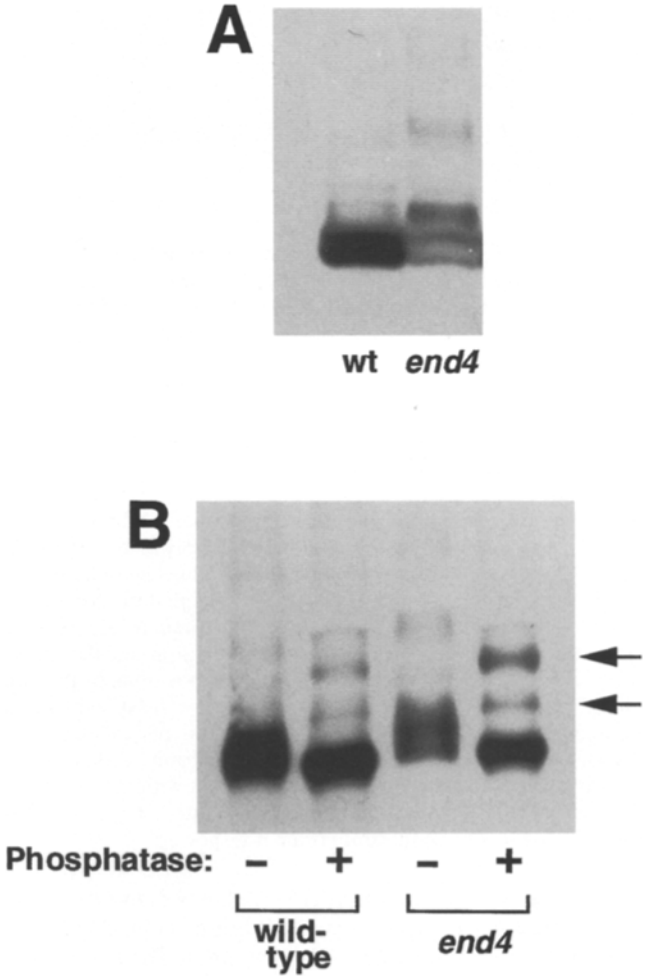

Figure 4. The effect of *end4* on receptor phosphorylation and ubiquitination. Protein extracts were prepared from the wild-type *END4* (NDY356) and mutant *end4* (NDY359) isogenic pair of *MATα GAL1-STE3 pep4Δ* strains cultured as described for Fig. 3. (*A*) Effect of *end4* on receptor phosphorylation levels. The two extracts were analyzed by Western analysis using the anti-Ste3p antibody. (*B*) Effect of *end4* on receptor ubiquitination. Extracts were treated with potato acid phosphatase (+) or were mock treated (−), and then subjected to SDS-PAGE followed by Western analysis with the anti-Ste3p antibody. The positions of the two phosphatase-resistant, ubiquitinated receptor species evident upon phosphatase digestion are indicated at right (*arrows*).

the same at 30°C (a temperature permissive for the growth of *end4-1* cells) and at 36°C (Fig. 3; data not shown). Similar impairment of endocytosis by this *end4* allele at permissive temperatures was also noted for the constitutive endocytosis of Ste6p (Kolling and Hollenberg, 1994; Berkower et al., 1994).

Trapping the receptor at the cell surface by the *end4* mutational block affects both phosphorylation and ubiquitination of the receptor. The mobility of the cluster of phosphorylated receptor species at 48 kD was found to be somewhat slower and more disperse for receptor isolated from *end4* cells (Fig. 4 *A*), indicating that the receptor in the *end4* background is subject to more extensive phosphorylation. Ubiquitinated receptor species were visualized after phosphatase treatment to remove heterogeneity in gel mobility due to phosphorylation. Clearly, both the presumptive mono- and di-ubiquitinated receptor forms were found to be more abundant for receptor extracted from *end4* vs wild-type cells (Fig. 4 *B*). Indeed, the di-ubiquitinated form was especially prominent for the *end4*-derived receptor. Blocking endocytosis, therefore, does not block receptor modification. Such a block would have been expected if modification occurred exclusively at some postsurface endocytic transport step. Instead, the effect of *end4* is to increase the level of these two modifications, suggesting that both modifications may occur while the receptor is surface-resident. Increasing the receptor's surface residency time would increase its availability for surface-localized modifications.

Cells Depleted for Ubiquitin Show Both Impaired Receptor Turnover and Receptor Retention at the Surface

Ubiquitination often functions to target short-lived proteins for degradation by the proteosome. Although Ste3p is quite short lived with a half-life of ~20 min, its turnover is wholly dependent on vacuolar proteases and not on the proteosome (Davis et al., 1993). Nonetheless, ubiquitination of Ste3p could play a role in signaling its rapid degradation. One possibility is that ubiquitin modification might mark surface receptor and target its endocytic transport to the vacuole. Alternatively, instead of affecting transport, ubiquitination could play an essential role in designating the endocytosed receptor as a substrate for the vacuolar

proteases, analogous to its role in designating cytoplasmic proteins as substrates for the proteosome. To test if ubiquitin might participate in receptor endocytosis and turnover, we have made use of a mutant strain background that allows cells to be depleted of the ubiquitin protein itself.

The strain NDY377, a *MATα* derivative of SUB312 (Finley et al., 1994), has all four of the wild-type chromosomal ubiquitin structural loci deleted or disrupted. The sole source of ubiquitin in these cells is a plasmid-borne ubiquitin–β-galactosidase fusion construct expressed from the regulatable *GAL1* promoter (pUB23). The ubiquitin fusion construct is processed in vivo to yield free ubiquitin (Bachmair et al., 1986). The intracellular pool of free ubiquitin in these cells, assessed by Western immunoblot with ubiquitin-specific antibodies, was found to be approximately twofold lower than in a wild-type strain under galactose growth conditions. Free ubiquitin levels were undetectable by our methods 5 or 6 h after the imposition of a glucose-mediated block to further synthesis from the *GAL1* promoter (data not shown). We have also made use of the isogenic strain NDY365 in which a plasmid-borne ubiquitin gene expressed from the *CUP1* promoter (pUB39) replaces the *GAL1*-driven construct. Expression from this promoter is glucose insensitive, and thus, these cells serve as a control wherein ubiquitin is not depleted, but instead is produced constitutively at high levels. The amount of free ubiquitin as assessed by Western blotting was found to be about five- to tenfold greater in NDY365 cells than in wild type (data not shown).

Ubiquitin is required for several cell-essential activities (Finley et al., 1994; Ciechanover, 1994; Hochstrasser, 1995). Nonetheless, after the glucose-mediated repression of new ubiquitin synthesis, NDY377 cells maintained exponential cell growth for several hours (Fig. 5). Eventually, with sufficient depletion of ubiquitin, cell growth ceased 6 to 7 h after glucose treatment (Finley et al., 1994; Fig. 5).

Effects of this ubiquitin-depletion regimen on the level of ubiquitin receptor modification were examined at various times after the imposition of the glucose-mediated block to new ubiquitin synthesis. In experiments with cells in which ubiquitin expression from the *GAL1*-ubiquitin-β-galactosidase plasmid is ongoing (e.g., before glucose addition), we found no difference in the proportion of **a**-factor receptors that were ubiquitin modified compared with receptor extracted from the *CUP1*–ubiquitin-expressing cells (data not shown). This was found to change after imposition of the block to new ubiquitin synthesis (Fig. 5, *inset*). 2 h after glucose addition, the proportion of receptors found to be modified with ubiquitin was significantly less in extracts from *GAL1*-ubiquitin-β-galactosidase cells than from the *CUP1*-ubiquitin control cells. After 4 h of ubiquitin depletion, no ubiquitin-modified receptor was detected (Fig. 5, *inset*).

To test if ubiquitin might participate in receptor endocytosis and turnover, we have examined the rate of receptor degradative turnover under the ubiquitin-depletion conditions. Turnover was examined during an interval 2.5–4 h after the imposition of the glucose-mediated block to new ubiquitin synthesis in NDY377 cells (*GAL1*-ubiquitin-β-galactosidase) or in NDY365 cells (*CUP1*-ubiquitin). This time frame was chosen as notable effects on receptor ubiquitination levels were seen during this interval (Fig. 5, *inset*), while the growth rate of the NDY377 cells remained unimpaired (Fig. 5). Cells were in vivo labeled for 10 min with [^{35}S]methionine, and then chased for up to 90 min with cold methionine. To follow the fate of newly synthesized receptor, extracts were prepared from the labeled cells at different chase time points, and the receptor protein was purified via immune precipitation (Fig. 6). For the NDY365 control cells, rapid Ste3p turnover was seen, consistent with the previously measured receptor half-life of ~20 min at 30°C (Davis et al., 1993). This contrasts sharply with the result observed for NDY377 cells. Under conditions of ubiquitin depletion, Ste3p turnover was substantially slowed; the receptor half-life was estimated to be ~2 h. Therefore, we conclude that receptor turnover depends on the level of available ubiquitin within the cell.

In addition to the effect on receptor turnover, it was also apparent in Fig. 6 that the depletion of ubiquitin also perturbs Ste3p expression, evident in terms of the decreased incorporation of label into Ste3p for the initial time point of the NDY377 cells (Fig. 6; compare intensity of Ste3p band at the 1-min time point between the *CUP1*-ubiquitin

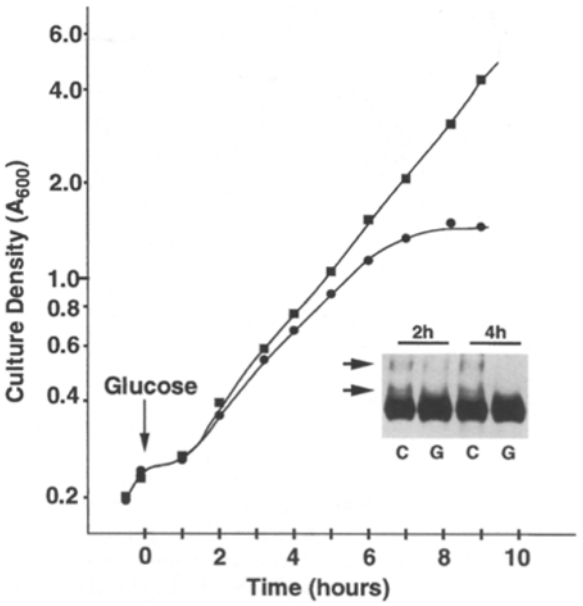

Figure 5. The effect of ubiquitin depletion on cell growth and on receptor modification. Ubiquitin-deletion strains, carrying plasmids either with the *GAL1*-ubiquitin-β-galactosidase (strain NDY377) or the *CUP1*–ubiquitin construct (strain NDY365), were grown to early log-phase in medium containing 2% galactose as the sugar source. To repress synthesis of ubiquitin from the *GAL1* promoter, 3% glucose was added (0 h). At 1-h intervals, culture aliquots were removed, and culture density was assessed through measurement of absorbance at λ_{600}. Growth curves are plotted as a function of time after glucose addition for strain NDY365 (*filled squares*) and for NDY377 (*filled circles*). (*Inset*) Effects on receptor ubiquitination. At the indicated times after glucose addition, culture aliquots from the ubiquitin-deletion strain carrying either the *GAL1*-ubiquitin-β-galactosidase plasmid (*G*) or the *CUP1*-ubiquitin plasmid (*C*) were removed, and protein extracts were prepared. Extracts were treated with potato acid phosphatase and subjected to gel electrophoresis and then Western analysis using the anti-Ste3p antibody. Positions of the ubiquitin-modified receptor species are indicated at left (*arrows*).

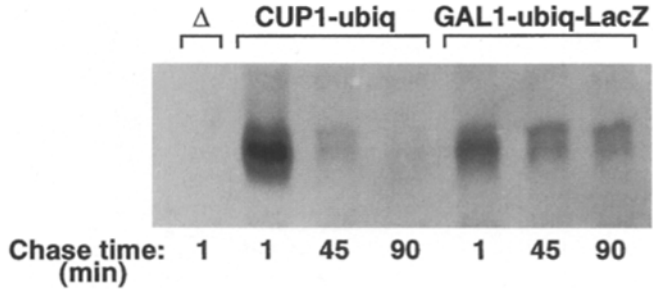

Figure 6. The effect of ubiquitin depletion on receptor turnover. The *MATα* ubiquitin-deletion strains, carrying plasmids either with the *CUP1*–ubiquitin construct (strain NDY365) or with the *GAL1*-ubiquitin-β-galactosidase construct (strain NDY377), were grown initially in minimal synthetic medium containing 2% galactose. Glucose was added to 3%, and then 2.5 h later, cells were labeled for 10 min with [^{35}S]methionine. Excess cold methionine and cysteine was then added, and cells were chased for times indicated. At this point, aliquots were removed and protein extracts were prepared. Extracts were immune precipitated with Ste3p-specific antiserum and subjected to SDS-PAGE, and the resulting purified Ste3p was visualized with autoradiography. As a control for proteins precipitated through cross-reaction with the Ste3p antiserum, an isogenic *MATa* strain (no Ste3p expression) SUB312 was processed in parallel.

and *GAL1*-ubiquitin-LacZ samples). This difference likely reflects effects on the rate of Ste3p synthesis, not Ste3p turnover: (*a*) loss of labeled receptor to vacuolar turnover is generally not a factor at such early timepoints, and (*b*) because these differences in Ste3p levels are retained when vacuolar turnover is blocked in *pep4*Δ cells (data not shown). Furthermore, this difference in Ste3p expression does not reflect a generalized impairment of protein synthesis caused by the ubiquitin depletion: the level of incorporation of [^{35}S]methionine both into total protein and into the cytoplasmic enzyme phosphoglycerol kinase was found to be unimpaired (data not shown). Therefore effects of the ubiquitin depletion on Ste3p expression likely are specific.

Turnover of **a**-factor receptor is by vacuolar proteases and requires endocytic transport of the receptor to the vacuole. The block imposed upon ubiquitin depletion could possibly result from a failed recognition of the receptor as substrate for vacuolar proteases, or alternatively, transport to the vacuole could be impaired. We have again made use of a whole cell proteolysis protocol to assess surface localization of the receptor under conditions of ubiquitin depletion. For these experiments, *pep4*Δ versions of the ubiquitin-delete strains were used, allowing assessment of localization in absence of any ongoing receptor degradation. Cells harboring either *GAL1*-ubiquitin-β-galactosidase plasmid (NDY426) or the *CUP1*-ubiquitin plasmid (NDY421) were pulse-labeled in vivo and chased as described above. As for the turnover experiment (Fig. 6), cell labeling was for 10 min and was initiated 2.5 h after the glucose-imposed block to new ubiquitin synthesis. At intervals after the pulse-labeling of newly synthesized receptor, culture aliquots were removed and treated with the energy poisons (10 mM NaN_3 and 10 mM KF) to block further transport. These intact cells were then subjected to digestion with added proteases. Only receptor that is exposed at the cell surface is susceptible to digestion; endocytosed receptor that has been removed from the surface resists digestion (Davis et al., 1993). After the protease digestion, extracts were prepared, and the labeled receptor was purified for gel electrophoresis by immune precipitation. As a control for the maintenance of cell integrity, we have also monitored by immune precipitation the digestion of the cytoplasmic enzyme phosphoglycerol kinase and found no evidence of digestion at any of the time points (data not shown).

In the control cells where ubiquitin is produced constitutively from the *CUP1* promoter, we found that the receptor was maximally at the surface 10 min after synthesis (Fig. 7). This was evident both in terms of the diminution of the receptor protein band (>50% susceptibility) and by the appearance of a digestion product at 30 kD, corresponding to the cytoplasmic tail plus seventh transmembrane domain (the Ste3p antiserum was raised against the cytoplasmic tail domain). At longer times after synthesis,

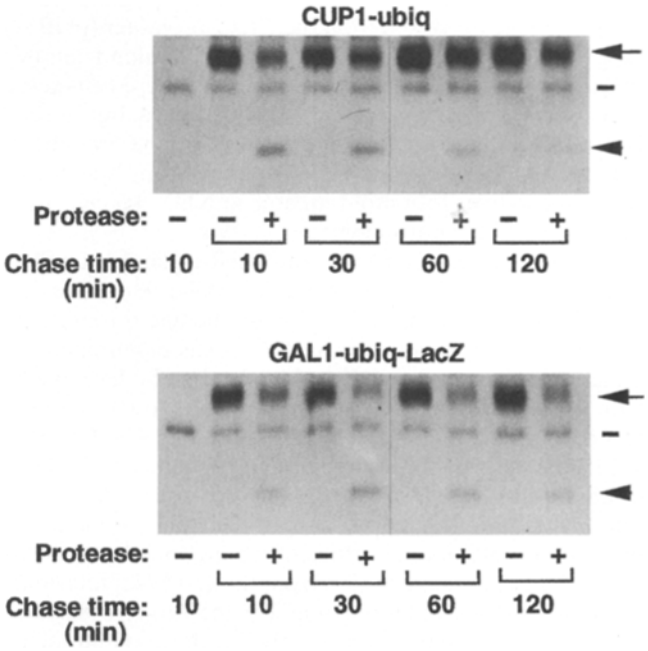

Figure 7. The effect of ubiquitin depletion on the endocytic removal of receptor protein from the cell surface. *pep4*Δ versions of the ubiquitin-deletion strain, carrying plasmids either with the *CUP1*–ubiquitin construct (strain NDY421) or with the *GAL1*-ubiquitin-β-galactosidase construct (strain NDY426), were grown and in vivo labeled with [^{35}S]methionine 2.5 h after a glucose-mediated block to new ubiquitin synthesis as described for Fig. 6. Samples were taken 10, 30, 60, and 120 min after the 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 immune precipitated using Ste3p-specific antiserum. To identify those proteins that precipitate due to cross-reaction with the antiserum, NDY422, an isogenic *ste3*Δ*::LEU2* derivative of NDY421 was processed in parallel. Results from strain NDY421 (*CUP1*-ubiquitin) are shown in the top panel, while those from NDY426 (*GAL1*-ubiquitin-β-galactosidase) are shown below. (*Right*) Positions of full-length Ste3p (*arrow*), the 30-kD CTD Ste3p digestion product (*arrowhead*), and a species cross-reactive with the Ste3p antiserum also precipitated from *ste3*Δ*::LEU2* cells (*hash mark*).

the receptor became increasingly less susceptible to the external proteases, consistent with its removal from the cell surface by endocytosis. Changing protease susceptibility presumably reflects changing localization for the receptor: cytoplasmic synthesis, delivery to the cell surface, and finally, endocytosis to the vacuole. Although the *CUP1*-ubiquitin control for these experiments constitutively overexpresses ubiquitin (five- to tenfold), the transient surface residency of Ste3p observed in these cells is consistent with kinetics of endocytosis, as previously assessed in strains with wild-type levels of ubiquitin. In such wild-type strains, receptor was found to be maximally available at the cell surface 10 to 30 min after synthesis (Davis et al., 1993).

Under ubiquitin-depletion conditions, i.e., in the *GAL1*-ubiquitin-β-galactosidase background (strain NDY426), newly synthesized receptor was again found to be largely surface localized at the earliest time point (Fig. 7; 10 min). At longer times after synthesis, however, obvious effects on receptor traffic were apparent (Fig. 7). At the 30-min time point, the receptor showed somewhat increased protease susceptibility relative to the first time point, consistent with further delivery of the newly synthesized receptor to the surface during this interval (10–30 min after synthesis). Once delivered to the surface, slight if any loss of susceptibility to the external proteases was evident over the next 90 min (Fig. 7; compare 30-, 60-, and 120-min time points). The bulk of the receptor population remained surface localized fully 2 h after its biosynthesis. Thus, under these ubiquitin-depletion conditions, the constitutive endocytosis of Ste3p, which is normally quite rapid, was found to be drastically impaired if not blocked altogether. Therefore we conclude that the normal rate of receptor constitutive endocytosis depends on the availibility of sufficient levels of intracellular ubiquitin.

Involvement of Two Ubiquitin-Conjugation Functions in Receptor Ubiquitination and Turnover

The E2 enzymes, encoded by the *UBC* genes in yeast, function in substrate recognition for ubiquitination and in the enzymatic addition of the ubiquitin moiety. 10 distinct *UBC* genes have been identified in yeast to date (Chen et al., 1993). This diversity in ubiquitin-conjugating enzymes likely reflects the diversity of substrate proteins that must be recognized for ubiquitination. We were interested to know if any of these *UBC* functions participated in the ubiquitination of the **a**-factor receptor. Work on another cell surface membrane protein, the **a**-factor exporter protein Ste6p had implicated *UBC4* and *UBC5* as being involved in the rapid vacuolar turnover of this protein (Kolling and Hollenberg, 1994). Ubc4p and Ubc5p are close sequence homologs and appear at least in part to be functionally redundant (Seufert and Jentsch, 1990). In *ubc4Δ ubc5Δ* cells, turnover of Ste6p that normally has a half-life of ~20 min was found to be slowed by two- to threefold (Kolling and Hollenberg, 1994).

In light of the above findings, we have asked if *UBC4* and *UBC5* might participate in the ubiquitination of Ste3p. Immunoblots of Ste3p extracted from either the *ubc4Δ ubc5Δ* cells or from cells of the isogenic wild-type strain showed several obvious differences. The *ubc4Δ ubc5Δ* strain showed a 2.5-fold over accumulation of the receptor protein as well as increased Ste3p phosphorylation (Fig. 8). Similar effects on both Ste3p quantity and phosphorylation were noted previously for *end4* cells (Fig. 4 *A*), suggesting that like *end4*, the *ubc4Δ ubc5Δ* mutations might exert their effects through impairment of **a**-factor receptor endocytosis. To examine the effect of these mutations on receptor ubiquitination, extracts were treated with phosphatase and diluted so that equivalent amounts of receptor could be compared. Clearly, the *ubc4Δ ubc5Δ* mutations lead to a significant decrease in the proportion of the receptor that is ubiquitin modified. This suggests that Ubc4p and Ubc5p may play a direct role in the recognition of Ste3p as a substrate for ubiquitination.

We have also investigated the involvement of the *UBC4/UBC5* functions in receptor turnover. Ste3p turnover was compared in three isogenic *MAT*α strains: (*a*) a wild-type strain, (*b*) a *pep4Δ* strain, and (*c*) a *ubc4Δ ubc5Δ* strain. Cells were labeled in vivo for 10 min with [^{35}S]methionine, and then chased with cold methionine for the times indicated (Fig. 9). In the wild-type background, the usual rapid turnover is apparent. In the *pep4* cells, turnover is blocked. In the *ubc4Δ ubc5Δ* background, turnover was found to be impaired, showing a receptor half-life of ~45 min—two- or threefold slower than in wild-type cells. Thus, decreased receptor ubiquitination is correlated with decreased receptor turnover, again suggesting a possible causal link between the two processes.

In addition to the *ubc4Δ ubc5Δ* double mutant, we have also tested Ste3p turnover in other *ubc* mutant backgrounds. Consistent with the functional redundancy of *UBC4* and *UBC5*, no effect on Ste3p turnover was observed in a *ubc4Δ ubc6Δ* mutant background (data not shown). Furthermore, a temperature-sensitive *cdc34 (ubc3)* strain showed no Ste3p turnover defect at its nonpermissive temperature (data not shown). In addition, cells deleted for the E3 function *UBR1* did not manifest impaired Ste3p turnover (data not shown).

a-Factor-induced Ubiquitination of the Receptor

In addition to ligand-independent, constitutive endocytosis, the **a**-factor receptor is also subject to ligand-depen-

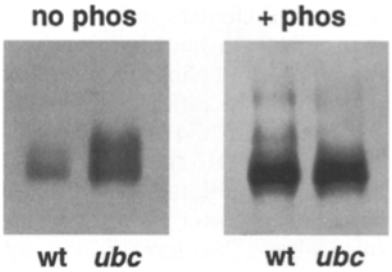

Figure 8. The effect of *ubc4Δ ubc5Δ* mutations on receptor modification. Protein extracts were prepared from the wild-type *MAT*α strain MHY501 and the isogenic *ubc4Δ ubc5Δ* strain MHY508. (*Left*) Samples are subjected to Western analysis with affinity-purified Ste3p-specific antibody. (*Right*) Extracts were treated with potato acid phosphatase, and the *ubc4Δ ubc5Δ* sample was diluted 2.5-fold to normalize levels of Ste3p for Western analysis.

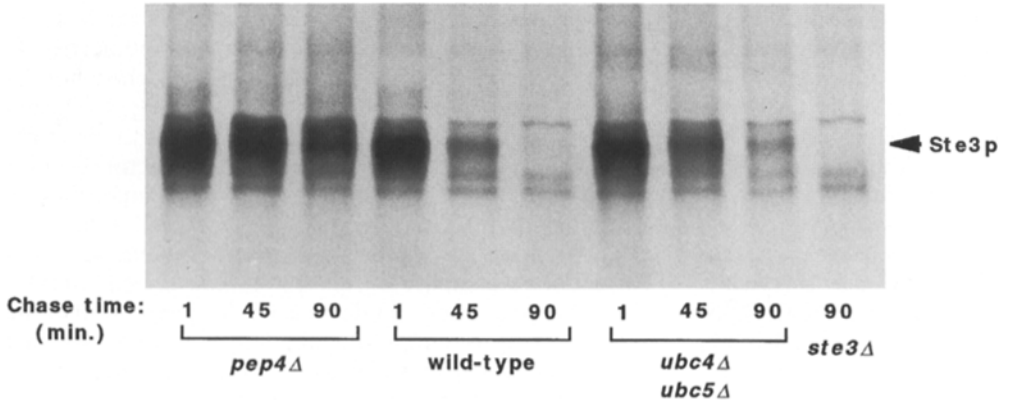

Figure 9. The effect of *ubc4Δ ubc5Δ* mutations on the turnover of the **a**-factor receptor. Cell cultures from three isogenic *MATα* strains were labeled in vivo for 10 min with [^{35}S]methionine: (*a*) wild-type (MHY501), (*b*) *pep4Δ::LEU2* (NDY346), or (*c*) *ubc4Δ ubc5Δ* (MHY508). Samples were taken 1, 45, and 90 min after initiation of the chase, and then extracts were prepared. Extracts were immune precipitated with Ste3p-specific antiserum and subjected to SDS-PAGE, and the resulting purified Ste3p was visualized with autoradiography. As a control for the cross-reaction of the Ste3p antiserum, an isogenic *ste3Δ* (NDY277) cell culture was labeled and processed in parallel (*ste3Δ*). The position of Ste3p is indicated at right (*arrow*). Proteins present in the *ste3Δ* lane are cross-reactive species that contaminate all the immune precipitations.

dent endocytosis (Davis et al., 1993). Ligand-dependent uptake is most conveniently studied using a mutant receptor protein that is wholly defective for rapid constitutive endocytosis. *STE3Δ365* encodes a truncated version of the receptor missing the COOH-terminal 105 amino acids of the 185-residue-long CTD. In the absence of added **a**-factor ligand, Ste3Δ365p does not undergo endocytosis, and instead it accumulates at the cell surface (Davis et al., 1993). However, when presented with **a**-factor, Ste3Δ365p was found to undergo internalization that, like the constitutive process, delivers receptor to the vacuole. We have exploited this mutant receptor to investigate the possible involvement of phosphorylation and ubiquitination in ligand-dependent endocytosis. Cells of the *MATα GAL1-STE3Δ365 pep4Δ* strain SY2635 were grown on galactose-containing medium for 2 h, allowing receptor expression and accumulation at the cell surface. 30 min after terminating new receptor synthesis with the addition of 3% glucose, cells were treated for 90 min with or without **a**-factor. Comparison of receptor protein extracted from pheromone-treated vs the mock-treated cells showed that the **a**-factor treatment led to increased phosphorylation of Ste3Δ365p (Fig. 10 *A*). Ligand-dependent phosphorylation generally observed for 7-TMS receptors is thought to play an adaptive role in regulating receptor activity (Dohlman et al., 1991).

To investigate ligand-dependent changes in ubiquitination, we have again used the epitope-tagged ubiquitin constructs in combination with potato acid phosphatase treatment. SY2635 cells carried one of three different plasmids: (*a*) empty plasmid (pND167), (*b*) plasmid containing the *CUP1*-driven wild-type ubiquitin (pND164), or (*c*) plasmid with the *CUP1*-driven *myc*-tagged ubiquitin (pND165). Cells were grown and treated with **a**-factor pheromone for 90 min exactly as described above. We have previously shown that this treatment results in quantitative endocytosis of Ste3Δ365p to the vacuole (Davis et al., 1993). Pheromone treatment induced two phosphatase-resistant modified receptor species at 38 and 45.5 kD (Fig. 10 *B*, *vector only* lanes). In the exposure shown, only the 38-kD species is evident. When cellular levels of ubiquitin were increased from the *CUP1*-ubiquitin plasmid pND164, the proportion of the receptor present as these modified species also increased (Fig. 10 *B*, *wt ubiq* lanes). For these cells, increased modification was seen even for the mock-treated (i.e, no **a**-factor) cells, where a modified species at 38 kD is evident. Phosphatase-resistant modification was again further increased with **a**-factor treatment; more of the 38-kD species was evident, as well as a 45.5-kD species. The finding that the extent of modification is sensitive to the level of ubiquitin available in the cell suggests that this **a**-factor–induced modification is in fact ubiquitin. This interpretation was solidified when the same experiment was performed in cells that overproduce the epitope-tagged ubiquitin (from pND165). In this case, modified receptor species with slightly slowed electrophoretic mobilities were evident (Fig. 10 *B*, *myc-ubiq* lanes), consistent with modification by the enlarged, 90-residue-long tagged ubiquitin. Therefore, we conclude that the **a**-factor–induced, phosphatase-resistant modification is indeed ubiquitination.

The apparent molecular weight of the two ubiquitin-modified forms of the receptor suggest that they represent the mono- and di-ubiquitinated forms. Most of the phosphatase-treated Ste3Δ365p migrates at 29.5 kD, while the two modified forms are at 38 and 45.5 kD. In cells expressing the *myc*-tagged ubiquitin, the lower of the two modified receptor forms is present as a doublet—one that migrates at 38 kD and one at the 39.5-kD position. This higher molecular weight species probably corresponds to the addition of a single *myc*-tagged ubiquitin, while the 38-kD species represents ubiquitination by endogenous, chromosomally expressed wild-type ubiquitin. At the di-ubiquitinated position, a triplet cluster of receptor species is evident (Fig. 10 *B*). This is to be expected for a protein modified with two ubiquitin moieties, as there are three combinations possible: (*a*) two wild-type ubiquitin moieties, (*b*) one wild-type and one *myc*-tagged ubiquitin, or (*c*) two *myc*-tagged ubiquitins.

Discussion

a-Factor Receptor Is Subject to Constitutive and Ligand-dependent Phosphorylation

The **a**-factor receptor is subject to two distinct covalent modifications: phosphorylation and ubiquitination. Phos-

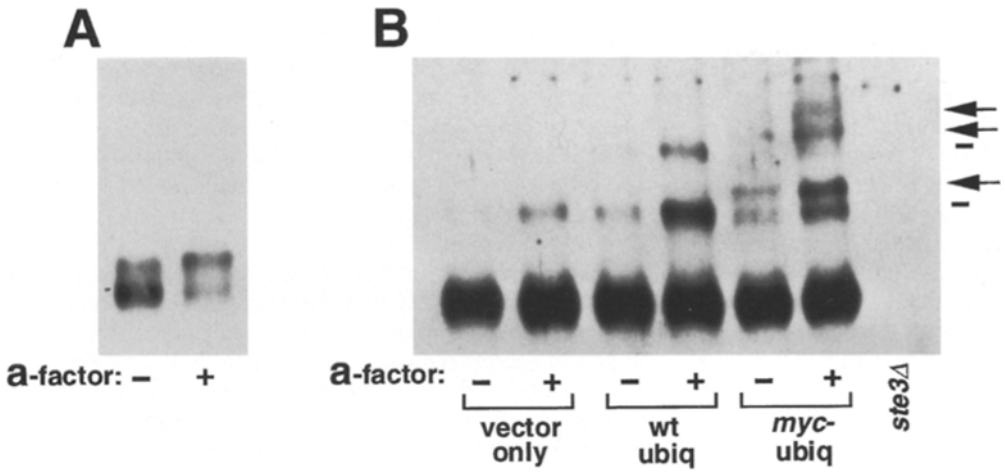

Figure 10. Assessment of the ligand-dependent phosphorylation and ubiquitination of the tail-truncated receptor, Ste3Δ365p. *MATα GAL1-STE3Δ365 pep4Δ* cells (SY2635), carrying one of three ubiquitin-expression plasmids were induced for ubiquitin expression with 100 μM $CuSO_4$ for 2.5 h: (*a*) pND164 with *CUP1*-driven wild-type ubiquitin (*wt*), (*b*) pND165 with *CUP1*-driven *myc*-tagged ubiquitin (*myc-ubiq*), or (*c*) the vector control plasmid pND167 that carries only the *CUP1* promoter but no downstream ubiquitin coding sequences (*vector only*). Subsequently, 2% galactose was added for 90 min to induce synthesis of the tail-truncated receptor Ste3Δ365p. New receptor synthesis was then repressed with 3% glucose for 30 min, and then cells were treated with **a**-factor (+**a**) or mock treated (−) for 90 min. (*A*) Effect of pheromone treatment on receptor phosphorylation levels. Extracts prepared from SY2635 cells carrying pND167, grown and exposed to **a**-factor as described above, were subjected to SDS-PAGE and Western analysis with the anti-Ste3p antibody. (*B*) Effect of pheromone treatment on receptor ubiquitination. Extracts from SY2635 cells, carrying the three ubiquitin expression plasmids cultured and pheromone treated as described above, were digested with phosphatase, and then subjected to SDS-PAGE and Western analysis with the anti-Ste3p antibody. The positions of the ubiquitin-modified receptor forms are indicated with bars at right, while the positions of modified forms that likely involve the *myc*-tagged ubiquitin are indicated with arrows.

phorylation of the serine/threonine-rich CTD has been demonstrated for a number of 7-TMS receptors (Dohlman et al., 1991), including the Ste3p counterpart, the α-factor receptor (Reneke et al., 1988; Zanolari et al., 1992). Herein we show that the **a**-factor receptor is subject to a constitutive or basal level of phosphorylation, and as with other 7-TMS receptors, ligand stimulation leads to increased phosphorylation. Ligand-induced phosphorylation occurs for the Δ365 tail-truncated receptor (Fig. 10) as well as for the wild-type **a**-factor receptor (Davis, N.G., unpublished results). For retinal rhodopsin and the β-adrenergic receptor, CTD phosphorylation is part of a mechanism for rapid adaptive regulation of the receptor's signaling activity (Dohlman et al., 1991). We would suggest, therefore, a similar role for phosphorylation in the regulation of the **a**-factor receptor. Consistent with such a role, we have previously demonstrated that **a**-factor receptors with a truncated CTD, and therefore, missing potential phosphorylation sites, show a heightened, supersensitive response to ligand (Boone et al., 1993). Based on similar findings, a role for CTD phosphorylation in receptor desensitization has also been suggested for the α-factor receptor (Reneke et al., 1988; Konopka et al., 1988; Chen and Konopka, 1996).

Involvement of Ubiquitination in Surface Protein Endocytosis and Turnover

Ubiquitination has been identified as a possible modification for surface membrane proteins both in mammalian cells (Siegelman et al., 1986; Yarden et al., 1986; Leung et al., 1987; Mori et al., 1992; Cenciarelli et al., 1992; Paolini and Kinet, 1993) and in yeast (Kolling and Hollenberg, 1994; Hicke and Riezman, 1996). For mammalian cell surface receptors, the function of this modification has remained uncertain. In yeast, four pieces of evidence now point to the involvement of ubiquitination in endocytosis. First, a multicopy plasmid carrying the yeast *UBI4* gene, encoding four tandem copies of the ubiquitin protein, was found to suppress lethality associated with a mutant allele of the clathrin heavy chain gene (Nelson and Lemmon, 1993). Overexpression of ubiquitin therefore compensates for some clathrin-associated defect. It is notable in this regard that a partial requirement for yeast clathrin in the endocytic uptake of the pheromone receptors has been demonstrated (Payne et al., 1988; Tan et al., 1993). Second, a possible role for ubiquitination in the endocytosis and vacuolar degradation of two yeast cell surface transporters has been suggested—the **a**-factor exporter protein, Ste6p, and the ATP-binding cassette multidrug transporter protein, Pdr5p (Kolling and Hollenberg, 1994; Egner and Kuchler, 1996). Like the **a**-factor receptor, both Ste6p and Pdr5p undergo a rapid, constitutive endocytosis that delivers surface protein to the vacuole for degradative turnover (Kolling and Hollenberg, 1994; Berkower et al., 1994; Egner et al., 1995), and both have found to be ubiquitinated (Kolling and Hollenberg, 1994; Egner and Kuchler, 1996). In addition, a threefold impairment in the rate of Ste6p turnover was observed in a *ubc4Δ ubc5Δ* mutant cell background (Kolling and Hollenberg, 1994). Third, work on the general amino acid permease Gap1p previously identified the gene *NPI1* as required for the regulated inactivation of surface permease activity (Grenson, 1983). Recent analysis of *NPI1* revealed that it is identical to *RSP5*, which apparently encodes a ubiquitin E3 function (Hein et al., 1995). In addition to effects on Gap1p activity, these authors also showed that the *npi1/rsp5* mutation impairs the vacuolar turnover of Fur4p, the uracil permease. Fi-

nally, a recent report on the α-factor receptor indicates an involvement for ubiquitin in ligand-dependent endocytosis of this receptor (Hicke and Riezman, 1996). They find both a ligand-dependent ubiquitination of the receptor and a substantial impairment of ligand uptake in the *ubc4 ubc5* mutant background. Most significantly, in terms of linking receptor ubiquitination to endocytosis, they find that a single lysine to arginine mutation in a severe COOH-terminally truncated version of the α-factor receptor simultaneously blocks receptor ubiquitination as well as the receptor-mediated uptake of the α-factor ligand.

These observations have now been extended to the endocytosis and turnover of the **a**-factor receptor. Our work that focuses on the constitutive uptake of this receptor complements the analysis of Hicke and Riezman (1996), which concentrated on the ligand-dependent uptake of α-factor receptor. Under conditions in which constitutive endocytosis is proceeding normally, ~10% of the receptor protein is present as ubiquitin-modified species (apparent either as the mono- or di-ubiquitinated forms). Furthermore, this low level of ubiquitination is seen to increase in an *end4* mutant cell background where the normal constitutive endocytosis of the receptor is blocked and the receptor accumulates at the cell surface. This suggests that ubiquitination may occur in wild-type cells during the window of time while the receptor is surface-resident.

To test for the potential involvement of ubiquitination in the constitutive endocytosis and turnover of the receptor, we have made use of cells in which all of the chromosomal copies of the ubiquitin structural gene have been deleted, and which survive by virtue of an episomal copy of the ubiquitin gene conditionally expressed from the *GAL1* promoter. The depletion of ubiquitin by glucose repression of the *GAL1* promoter results in very striking effects on both receptor turnover and localization. Receptor turnover is drastically impaired with its half-life increasing from 20 min to ~2 h. Accompanying the turnover effects is a dramatic change in localization. In wild-type cells, residency of the **a**-factor receptor at the surface is short lived, being maximally present at the surface 10 to 30 min after synthesis (Davis et al., 1993). Under conditions of ubiquitin depletion, the majority of the receptor was found to be surface retained even 2 h after its synthesis. Therefore, it appears that the impaired turnover of the receptor is a consequence of its impaired endocytosis.

Based on results with the α-factor receptor, Hicke and Riezman (1996) have suggested that the ligand-dependent ubiquitination of this receptor is a requirement for initiating its ligand-dependent endocytic uptake. Consistent with this, using the truncation mutant *STE3Δ365*, we see a ligand-dependent ubiquitination that accompanies ligand-stimulated uptake. The bulk of our analysis, however, has focused on ligand-independent processes, and based on these results, we would like to suggest that ubiquitin may play an essential role in initiating constitutive uptake as well. Conceptually, attachment of ubiquitin provides a way of marking surface receptor, providing a molecular flag for recognition by the cell's endocytic apparatus. In this scenario, the under-ubiquitination of the receptor that accompanies ubiquitin depletion would be insufficient to trigger uptake. We cannot eliminate the possibility that the effects of ubiquitin depletion on receptor endocytosis are indirect. For instance, some component of the endocytic apparatus could, in theory, be regulated through ubiquitination. Furthermore, since ubiquitin is an essential protein, its depletion eventually leads to diverse cellular processes being shutdown—among these may be endocytosis. It is notable in this regard that our measurements of receptor turnover and endocytosis occurred relatively soon after the imposition of the glucose-mediated block to new ubiquitin synthesis. At this time, cells still showed exponential growth kinetics as well as unimpaired protein synthesis. It is unlikely that the ubiquitin depletion results in a generalized impairment of cellular membrane traffic; impaired secretory flow of membrane would be expected to have a fairly direct and immediate effect on cell growth kinetics. Furthermore, the protease-susceptibility protocol used to measure receptor residency times (Fig. 7) can also be used to assess the kinetics with which newly synthesized receptor is delivered to the surface. In such experiments, rates of appearance of the newly synthesized receptor at the cell surface, a measure of secretory pathway function, were found to be roughly equivalent in the *GAL1*-ubiquitin and control strains under the glucose-repression conditions (data not shown). Therefore, the effects of ubiquitin depletion appear to be specific for the endocytic trafficking of the receptor.

Involvement of Ubiquitin E2 and E3 Activities in the Ubiquitination of Cell Surface Proteins

In addition to the ubiquitin structural genes and the yeast E1 activity (encoded by UBA1), which is required for the initial activation of newly synthesized ubiquitin, the cell expresses a large number of additional enzymatic activities, E2 and E3 enzymes, that mediate both substrate recognition and the actual conjugation of the ubiquitin moieties to the substrate protein. The diverse number of these activities is thought to reflect the diversity of substrates that the ubiquitin system must normally recognize. Which of these are involved in the ubiquitination of the pheromone receptors? To date, 10 distinct *UBC* genes encoding E2 activities have been identified (Chen et al., 1993). Our data on the ubiquitination of Ste3p suggest a central role for Ubc4p and Ubc5p. *ubc4Δ ubc5Δ* cells show severe diminution of Ste3p ubiquitination and also slowed Ste3p turnover. Furthermore, *ubc4Δubc5Δ* cells are also partially defective for the rapid turnover of the **a**-factor exporter protein Ste6p (Kolling and Hollenberg, 1994) and for the endocytic uptake of α-factor (Hicke and Riezman, 1996). *ubc* mutants that were found to have no effect on Ste3p turnover include the *ubc4Δ ubc6Δ* double mutant as well as *ubc3(cdc34)*. Analysis of the endocytic uptake of α-factor also showed, in addition to *UBC4* and *UBC5*, a partial requirement for *UBC1*, while no effect of *ubc2*, *ubc6*, *ubc7*, or *ubc8* mutations was evident (Hicke and Riezman, 1996).

Three E3 activities have so far been identified: the E3 for the N-end rule pathway (encoded by *UBR1*) (Bartel et al., 1990), as well as two genes showing strong homology to the mammalian E3 activity, E6-AP, these being *UFD4* and *RSP5* (Johnson et al., 1995; Huibregtse et al., 1995). No effect on Ste3p turnover was apparent in *ubr1Δ* cells (data not shown). As mentioned previously, *RSP5* is now impli-

cated in the turnover of two other yeast cell surface proteins, the general amino acid permease and the uracil permease (Hein et al., 1995). The mammalian E3, E6-AP (Rsp5p homolog), normally acts together with an E2 that bears strongest homology to the yeast Ubc4p and Ubc5p (Scheffner et al., 1994), suggesting that Rsp5p, together with the E2s Ubc4p and Ubc5p, may act to regulate the endocytosis of yeast cell surface proteins. We are investigating the participation of *RSP5* in the ubiquitination and endocytosis of Ste3p.

Level of Receptor Ubiquitination

The ubiquitin-depletion experiments showed very strong effects on both receptor endocytosis and turnover, consistent with the entire cell surface receptor population being subject to a ubiquitin-dependent uptake. Yet, analysis of receptor modification consistently showed only a small minority of the receptors (~10%) with conjugated ubiquitin. This level of ubiquitination is consistent with what has been observed for other proteins known to rapidly turn over by a ubiquitin-dependent proteosomal mechanism. In these cases, the highly ubiquitinated species fail to accumulate, likely because of their rapid loss to proteosomal degradation. In the case of Ste3p, however, no obvious increase in the proportion of ubiquitinated receptor is seen even when turnover is blocked in *pep4Δ* cells. One possibility is that ubiquitin's participation in endocytosis is transient. Although ubiquitin may mark surface receptor for uptake, once uptake is initiated, perhaps ubiquitin is removed. Indeed, it is well documented that not only is ubiquitin continuously added to substrate proteins by E2 and E3 enzymes, but it is also subject to continuous removal by isopeptidase activities (Finley and Chau, 1991). In endocytosis, such isopeptidase activities may operate at a downstream transport step to remove ubiquitin moieties once endocytosis is initiated. Alternatively, receptor ubiquitin levels may be low for artifactual reasons. While ubiquitin addition is ATP dependent, removal is not. Therefore, some loss of ubiquitin modification could attend the preparation of protein extracts from whole cells.

A number of cell surface proteins have now been shown to undergo ubiquitination. The functional role of this modification has remained uncertain. Data presented in this paper suggest a likely role for ubiquitination in the endocytosis and turnover of the **a**-factor receptor. It will be interesting to see how generalized these findings will be for the metabolism of surface proteins in yeast and mammalian cells. Is this a feature of all receptor-mediated endocytosis, a feature only of vacuole-directed (lysosome-directed) endocytic transport, or a feature of a specialized mode of endocytosis, perhaps a clathrin-independent mechanism?

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