Monoubiquitin carries a novel internalization signal that is appended to activated receptors

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Ubiquitin modification of signal transducing receptors at the plasma membrane is necessary for rapid receptor internalization and downregulation. We have investigated whether ubiquitylation alters a receptor cytoplasmic tail to reveal a previously masked internalization signal, or whether ubiquitin itself carries an internalization signal. Using an α -factor receptor–ubiquitin chimeric protein, we demonstrate that monoubiquitin can mediate internalization of an activated receptor that lacks all cytoplasmic tail sequences. Furthermore, fusion of ubiquitin in-frame to the stable plasma membrane protein Pma1p stimulates endocytosis of this protein. Ubiquitin does not carry a functional tyrosine- or di-leucine-based internalization signal. Instead, the three-dimensional structure of the folded ubiquitin polypeptide carries an internalization signal that consists of two surface patches surrounding the critical residues Phe4 and Ile44. We conclude that ubiquitin functions as a novel regulated internalization signal that can be appended to a plasma membrane protein to trigger downregulation.

Keywords: endocytosis/internalization signal/protein targeting/receptor downregulation/ubiquitin

Introduction

Proteins that undergo regulated internalization must either carry an internalization signal that can be activated, or the protein must be modified with a signal in response to environmental cues. One example of an induced protein modification that regulates internalization is the phosphorylation that occurs on the G protein-coupled β -adrenergic receptor in response to ligand binding. Phosphorylated serines in the receptor cytoplasmic tail provide binding sites for arrestin, a protein that serves as a specialized adaptor to link the activated receptor to endocytic machinery (reviewed in Krupnick and Benovic, 1998).

Ubiquitylation is another modification that can regulate receptor internalization (reviewed in Bonifacino and Weissman, 1998; Hicke, 1999). Ubiquitin is a 76-amino-acid polypeptide that is conjugated to substrate proteins through the formation of an isopeptide bond between the ϵ amino group of lysine residues in target proteins and the C-terminal glycine of ubiquitin. Although ubiquitylation of

cytosolic proteins and endoplasmic reticulum membrane proteins generally serves as a recognition tag for destruction by the proteasome, ubiquitylation of plasma membrane proteins can serve to target their entry into the endocytic pathway.

In *Saccharomyces cerevisiae*, G protein-coupled signaling receptors, as well as permeases and transporters, are ubiquitylated at the cell surface (Hicke, 1999 and references therein). The α -factor (Ste2p) and a-factor (Ste3p) receptors bind mating pheromones and the receptor–pheromone interaction initiates a signal transduction pathway that leads to changes in the yeast cell that are required for mating. Upon ligand binding, the receptors are rapidly internalized and transported to the lysosome-like vacuole, where they are permanently inactivated by degradation (Davis *et al.*, 1993; Schandel and Jenness, 1994). Pheromone binding stimulates phosphorylation and ubiquitylation of Ste2p and Ste3p cytoplasmic tails, and both modifications are required for rapid receptor internalization (Reneke *et al.*, 1988; Hicke and Riezman, 1996; Roth and Davis, 1996). A 9-amino-acid sequence in the Ste2p tail, SINNDAKSS, promotes internalization (Rohrer *et al.*, 1993; Hicke and Riezman, 1996). The serines within this sequence are required for phosphorylation of the receptor; phosphorylation then positively regulates ubiquitylation at the SINNDAKSS lysine (Hicke *et al.*, 1998). Lysine residues in the α -factor receptor tail are critical components of receptor internalization signals because they serve as sites of ubiquitin conjugation (Hicke and Riezman, 1996; Terrell *et al.*, 1998). Unlike recognition and degradation by the proteasome, which generally require the formation of a polyubiquitin chain at least four subunits long, rapid Ste2p internalization occurs after monoubiquitylation of the receptor at a single lysine residue. Furthermore, the fusion of monoubiquitin in-frame to a receptor tail lacking all post-translational ubiquitylation sites is sufficient to promote internalization (Terrell *et al.*, 1998).

Ubiquitin-dependent internalization has also been demonstrated or implicated for cell surface proteins in mammalian cells. A number of proteins that are degraded in the lysosome are ubiquitylated at the cell surface, including the epidermal growth factor (EGF) receptor, the growth hormone receptor and the epithelial Na⁺ channel (Bonifacino and Weissman, 1998). Ubiquitylation and internalization of several growth factor receptors require the Cbl protooncogene, a ubiquitin protein ligase (Miyake *et al.*, 1998; Joazeiro *et al.*, 1999; Lee *et al.*, 1999; Waterman *et al.*, 1999). Similarly, degradation of the epithelial Na⁺ channel in the lysosome requires ubiquitylation by the ubiquitin protein ligase Nedd4 (Staub *et al.*, 1997). The growth hormone receptor undergoes ligand-stimulated ubiquitylation and cellular ubiquitylation machinery is also required for its ligand-stimulated

Fig. 1. Cytoplasmic tail variants of Ste2p. The cytoplasmic tail of Ste2p (residues 298–431) is represented schematically. Filled circles, lysine ubiquitylation sites; open circles, lysine→arginine mutations; TMD, transmembrane domain; (g)₁₅, Gly/Ser linker; UB, ubiquitin.

internalization. However, in this case ubiquitylation of the receptor itself may not be required (Strous *et al.*, 1996, 1997; Govers *et al.*, 1999).

The mechanism by which ubiquitin directs receptor internalization is unknown. Ubiquitylation of a receptor tail may trigger endocytosis by inducing a structural change in the tail that exposes a previously masked internalization signal. Alternatively, the ubiquitin polypeptide itself may carry internalization information that is appended to an activated receptor. To distinguish between these models we have defined the *cis*-acting information that is required for ubiquitin-dependent internalization. We have used plasma membrane protein–ubiquitin chimeric proteins to show that receptor tail sequences are not required for internalization. Instead, ubiquitin itself carries the information necessary to promote internalization of cell surface proteins. The internalization signal within ubiquitin is not a linear peptide sequence, but consists of two patches on the three-dimensional surface of the polypeptide in which Phe4 and Ile44 are the crucial residues.

Results

Truncated receptors fused to ubiquitin internalize α -factor rapidly

The α -factor receptor carries two strong ubiquitylation sites in its cytoplasmic tail (see Figure 1). These residues, Lys337 and Lys374, can individually mediate rapid rates of ligand-stimulated receptor internalization (M.Houlberg, S.C.Shih and L.Hicke, unpublished data). Receptors truncated following each of these sites, at amino acid 340 or 378, undergo ligand-stimulated internalization that depends on lysines present in the tail (Figure 2A; Terrell *et al.*, 1998). We have previously shown that ubiquitin fused in-frame to Ste2p at amino acid 376 (Ste2p-376–

Ub, Figure 1) can rescue internalization of the receptor lacking its ubiquitylation sites (Terrell *et al.*, 1998). To determine whether ubiquitin could also rescue internalization of a more severely truncated receptor, we fused ubiquitin to the receptor at amino acid 340 immediately following the SINNDKSS³³⁷SS sequence (Ste2p-340–Ub, Figure 1). Ste2p-340–Ub carried a mutation of Lys337 to Arg, to eliminate its only post-translational ubiquitylation site [Lys304 is not ubiquitylated (Hicke and Riezman, 1996)]. Ubiquitin was fused to receptors following a 15-amino-acid Gly/Ser linker that was included to allow independent folding of the receptor tail and ubiquitin domains (Robinson and Sauer, 1996). In addition, Lys48 of ubiquitin was mutated to arginine to remove the primary site of polyubiquitin chain formation, thereby preventing potential degradation of the chimeric protein during transit through the biosynthetic pathway. Chimeric receptors containing a ubiquitin moiety fused either at amino acid 340 or 376 rapidly internalized α -factor (Figure 2A). This internalization was directed by the fused ubiquitin moiety, not by ubiquitin that may become conjugated to one of the seven lysine residues of the fused ubiquitin, because internalization was also rapid when ubiquitin lacking all its lysines is fused to the receptor in the same way (Terrell *et al.*, 1998). Furthermore, fused ubiquitin was not conjugated to another ubiquitin moiety through its C-terminus because mutating or deleting the ubiquitin C-terminal glycines (Gly75 and Gly76) that are important for isopeptide bond formation had no effect on internalization of the chimeric receptor (unpublished data).

Internalization of α -factor by Ste2p is dependent on a number of proteins identified in genetic screens for endocytosis mutants (Raths *et al.*, 1993; Munn and Riezman, 1994; Munn *et al.*, 1995; Wendland *et al.*, 1996). To ensure that the Ste2p–Ub chimeric proteins internalized α -factor by the same pathway as wild-type Ste2p, we tested whether rapid internalization of pheromone by Ste2p-376–Ub was dependent on the End3, End4 and Pan1 proteins. *end3-1*, *end4-1* and *pan1-20* temperature-sensitive mutant strains expressing Ste2p-376–Ub or Ste2p-340–Ub were unable to internalize α -factor rapidly at the non-permissive temperature of 37°C (Figure 2B; K.E.Sloper-Mould and L.Hicke, unpublished data). These data indicate that similar endocytic machinery is required to internalize wild-type Ste2p and the receptor–ubiquitin chimeras. In addition, they demonstrate that End3p, End4p and Pan1p function to promote endocytosis downstream of receptor ubiquitylation.

Ubiquitin carries information sufficient for internalization

In Ste2p-340–Ub, ubiquitin is fused to the receptor immediately after the SINNDKSS internalization signal. The SINNDKSS sequence is the only information necessary for internalization of the truncated receptor Ste2p-345Stop because deletion of residues upstream of SINNDKSS in this truncated receptor has little or no effect on α -factor internalization (Rohrer *et al.*, 1993). The SINNDKSS sequence contains a strong ubiquitylation site at Lys337 and three serine residues that are required for phosphorylation of Ste2p-345Stop (Hicke *et al.*, 1998). To determine whether Ste2p phosphorylation is required in addition to ubiquitylation to mediate rapid internalization, we mutated

Fig. 2. Ubiquitin fused in-frame to Ste2p at amino acids 340 or 376 directs internalization of Ste2p-Ub chimeric proteins. All curves represent the average of at least three independent experiments and error bars depict the standard deviation. (A) Ste2p and Ste2p-Ub variants were introduced into *ste2* Δ cells and the resulting strains were assayed for their ability to internalize 35 S-labeled pheromone. Cells were harvested and incubated with [35 S] α -factor at 4°C, unbound α -factor was removed, and casamino acid medium pre-warmed to 30°C was added to initiate internalization. At various times aliquots of the cells were removed and the ratio of internal to total cell-associated α -factor was measured. Internalization half-times were determined by a simple exponential curve fit using Kaleidagraph software. Ste2p-376-Ub (LHY558, ●; $t_{1/2}$ = 9.2 min); Ste2p-340-Ub (LHY844, ▲; $t_{1/2}$ = 12.8 min); Ste2p-340Stop (LHY847, △; $t_{1/2}$ = 28.3 min); Ste2p-337R,340Stop (LHY319, ◇; $t_{1/2}$ > 200 min). (B) Wild-type (LHY558, ○), *end3-1* (LHY1488, ■), *end4-1* (LHY1490, ●) or *pan1-20* (LHY1580, ◆) strains expressing Ste2p-376-Ub (LHP361) as their sole source of Ste2p were propagated in casamino acid medium and shifted to the non-permissive temperature of 37°C for 20 min. 35 S-labeled α -factor was added and the ratio of internalized to total cell-associated α -factor was determined at different time points.

the three SINNDKSS serines to alanines in Ste2p-340-Ub. Mutating these serines had little effect on internalization of α -factor by the chimeric receptor (S.C. Shih and L. Hicke, unpublished data). To determine whether any other SINNDKSS residues are required together with ubiquitin to mediate Ste2p internalization, we created a chimeric receptor that carried ubiquitin fused at amino acid 321, upstream of SINNDKSS (Ste2p-321-Ub, see Figure 1). Ste2p simply truncated at amino acid 320 did not internalize α -factor and carried no sequences important for internalization (Rohrer *et al.*, 1993; Figure 3A). In contrast, Ste2p-321-Ub internalized α -factor as rapidly as Ste2p-340-Ub (Figure 3A). These data demonstrate that the SINNDKSS sequence and surrounding residues are not required for internalization of a receptor that carries a ubiquitin moiety.

Fig. 3. Ubiquitin is sufficient to internalize Ste2p. (A and B) α -factor internalization assays were performed on cells expressing Ste2p mutants as described for Figure 2A. All curves represent the average of at least three independent experiments and error bars represent the standard deviations. Internalization half-times were determined by an exponential curve fit using Kaleidagraph software, except for Ste2p-312-SINNDKSS, which required a linear curve fit. (A) Ste2p-340-Ub (LHY844, ■; $t_{1/2}$ = 12.8 min); Ste2p-320Stop (LHY852, ◇; $t_{1/2}$ > 200 min); Ste2p-321-Ub (LHY1194, ●; $t_{1/2}$ = 18.5 min). (B) Ste2p-303-Ub (LHY1212, ●; $t_{1/2}$ = 57.6 min); Ste2p-312-SINNDKSS (LHY1078, ○; $t_{1/2}$ = ~76 min). The fused ubiquitin moiety in Ste2p-303-Ub and the lysine ubiquitin acceptor site in Ste2p-312-SINNDKSS were approximately the same distance from the plasma membrane in these mutants (see Figure 1). (C) Assays that measure the number of α -factor binding sites present at the cell surface at various times after internalization at 30°C in the presence or absence of pheromone were performed on cells expressing different Ste2p variants (see Materials and methods). Ste2p-4xR,378Stop (LHY319, no α -factor, ●); Ste2p-378Stop (LHY18, no α -factor, ◆); Ste2p-376-Ub (LHY558, no α -factor, ■; + α -factor, □).

Ste2p truncated further upstream at amino acid 312 carries only 14 amino acids of its cytoplasmic tail and is unable to internalize α -factor. Fusing the SINNDKSS ubiquitylation motif to the severely truncated Ste2p-312Stop receptor can restore internalization (Rohrer *et al.*,

1993). Although internalization of Ste2p-312-SINNDKSS is relatively slow, it is absolutely dependent on the SINNDKSS lysine residue, demonstrating that fusion of a post-translation ubiquitylation site just 20 amino acids distal to the transmembrane domain can mediate internalization (Rohrer *et al.*, 1993). To test whether a ubiquitin moiety is sufficient for receptor internalization we fused ubiquitin to Ste2p at amino acid 303. Together with its Gly/Ser linker, this places ubiquitin approximately as far from the last transmembrane domain of the receptor as ubiquitin that is post-translationally conjugated to Ste2p-312-SINNDKSS, but the Ste2p-303-Ub chimera carries just six amino acids of the receptor tail (see Figure 1). Figure 3B shows that the Ste2p-303-Ub chimera internalized α -factor, indicating that ubiquitin can promote receptor internalization in the absence of almost the entire receptor tail.

Wild-type α -factor receptors are ubiquitylated and internalized at a slow rate in the absence of pheromone (Hicke *et al.*, 1998); however, a truncated receptor that lacks ubiquitylation sites is not constitutively internalized (Figure 3C). We tested whether the fusion of ubiquitin was sufficient to induce constitutive internalization of this truncated receptor. To assay constitutive internalization of a receptor-ubiquitin chimera we measured the clearance of α -factor binding sites from cells expressing Ste2p-376-Ub. In the absence of pheromone, Ste2p-376-Ub was internalized slightly faster than Ste2p-378Stop, which required post-translational ubiquitylation (Figure 3C). This observation indicates that the presence of a ubiquitin moiety fused to a receptor is sufficient to induce receptor endocytosis even in the absence of α -factor. The addition of ligand further stimulated the internalization of Ste2p-376-Ub (Figure 3C), indicating that ubiquitylation is not the only ligand-stimulated event that promotes internalization. The ligand-induced increase in the internalization rate may result from the relief of steric hindrance due to dissociation of the receptor-associated heterotrimeric G protein. Alternatively, ligand stimulation may lead to modifications of components of the endocytic machinery that enhance their activity, as has been demonstrated in mammalian cells (Slepnev *et al.*, 1998; Wilde *et al.*, 1999).

Fusion of ubiquitin can induce the endocytosis of a stable plasma membrane protein

If ubiquitin is a sufficient signal for endocytosis, then it should be able to direct internalization of a heterologous plasma membrane protein. Pma1p is an abundant and stable plasma membrane proton ATPase with a half-life of >11 h (Benito *et al.*, 1991; Figure 4). Previously, it was shown that the fusion of sequences that serve as ubiquitylation motifs from the Ste6 peptide transporter or the α -factor receptor could induce rapid internalization and degradation of Pma1p from the cell surface (Kölling and Losko, 1997; Roth *et al.*, 1998). We fused ubiquitin to the C-terminal amino acid of a hemagglutinin (HA)-epitope-tagged version of Pma1p under control of the glucose-repressible *GALI* promoter. The levels of Pma1p and Pma1p-Ub were analyzed at various times after the repression of new synthesis by incubation of cells in glucose. Figure 4A shows that Pma1p-Ub was degraded more rapidly than Pma1p. Quantification of these data indicated that appending ubiquitin to Pma1p decreased its

Fig. 4. Ubiquitin fused to the C-terminus of Pma1p increases its turnover rate. Strains carrying *GAL-HA-PMA1* or *GAL-HA-PMA1-UBI* plasmids were grown to log phase in casamino acid medium containing 2% galactose and 1% raffinose to induce protein expression from the *GALI* promoter. The cells were harvested, resuspended in glucose-containing medium to inhibit Pma1p or Pma1p-Ub synthesis, and allowed to recover for 20 min at 30°C. An aliquot of cells was removed immediately (time = 0 h) and additional aliquots were removed after 2, 3 and 4 h. Cell lysates were prepared, and proteins were separated by SDS-PAGE on 6% gels and analyzed by immunoblotting with anti-HA antiserum. The immunoblots shown were developed with chemiluminescence and half-times of degradation were calculated from blots of the same lysates developed with chemifluorescence to ensure that the signal was linear. (A) Degradation of Pma1p and Pma1p-Ub expressed in wild-type cells. (B) Degradation of Pma1p and Pma1p-Ub turnover shown in (A) was quantified. The curves represent the average of three independent experiments and error bars represent the standard deviations.

half-life from 11 to 3 h (Figure 4B). To confirm that Pma1p-Ub was degraded in the vacuole after being internalized from the cell surface, and not by the proteasome in the biosynthetic pathway, we expressed Pma1p and Pma1p-Ub in *pep4 prb1* cells that lack active vacuolar hydrolases (Klionsky *et al.*, 1990) and in *pre1 pre2* cells that carry defective proteolytic subunits of the proteasome (Heinemeyer *et al.*, 1991, 1993). Figure 5A and B shows that the degradation of HA-Pma1p-Ub depended on active vacuolar hydrolases but not on the proteasome.

Since one mutant form of Pma1p, Pma1-7p, can move directly from the Golgi to the vacuole and is degraded without traveling to the cell surface (Chang and Fink, 1995), we asked whether degradation of Pma1p-Ub required transport to and internalization from the plasma membrane. Mutations in the *SEC6* gene block transport of proteins from secretory vesicles to the plasma membrane but do not affect the transport of proteins that arrive at the vacuole through intracellular pathways. In particular, the degradation of Pma1-7p is not inhibited in a *sec6* mutant (Chang and Fink, 1995). Figure 5C shows that Pma1p-Ub degradation was inhibited in *sec6* cells incub-

Fig. 5. Pma1p-Ub is degraded in the vacuole after internalization from the cell surface. Pma1p and Pma1p-Ub were expressed under control of the *GAL1* promoter in different strains. The turnover rate of the Pma1p proteins was measured as described in Figure 4, except that cells were shifted for 20 min to the non-permissive temperature before the first aliquot of cells was withdrawn. (A) Degradation of Pma1p-Ub in *pep4 prb1* cells (LHY1479, $t_{1/2}$ = 15 h) and congenic wild-type cells (LHY1477, $t_{1/2}$ = 2.8 h) incubated at 37°C. (B) Degradation of Pma1p-Ub in *pre1 pre2* cells (LHY1473, $t_{1/2}$ = 2.9 h) and congenic wild-type cells (LHY1471, $t_{1/2}$ = 4.2 h) incubated at 38°C. (C) Degradation of Pma1p-Ub in *pan1-20* cells (LHY1469; $t_{1/2}$ = 13.4 h), *sec6* cells (LHY1487; $t_{1/2}$ = 11.5 h) and wild-type cells (LHY1465; $t_{1/2}$ = 3 h) incubated at 37°C. The immunoblots shown were developed with chemiluminescence and half-times of degradation were calculated from blots of the same lysates developed with chemifluorescence to ensure that the signal was linear.

ated at the non-permissive temperature. A similar result was observed in other mutants, *sec1* and *sec4* cells, that block the fusion of transport vesicles with the plasma membrane (S.C.Shih and L.Hicke, unpublished data). These data indicate that Pma1p-Ub must reach the cell surface to be degraded, suggesting that degradation occurs after internalization from the plasma membrane and subsequent transport to the vacuole. Furthermore, Pma1p-Ub degradation was blocked in *pan1-20* cells (Figure 5C), which are defective in the endocytosis of wild-type Ste2p and Ste2p-Ub but deliver proteins normally from the biosynthetic pathway to the vacuole (Wendland *et al.*, 1996; Figure 2B). Together, these experiments demonstrate that fusing ubiquitin to the C-terminus of Pma1p increased internalization of the protein from the cell surface.

Ubiquitin-dependent internalization is not mediated by a linear peptide signal but requires the three-dimensional structure of the folded ubiquitin polypeptide

Ubiquitin induces internalization of plasma membrane proteins to which it is attached. A simple explanation for this effect would be the presence in the ubiquitin polypeptide of a previously defined internalization signal, an aromatic residue- or di-leucine-based sequence. Transfer of

these sequences can induce internalization of heterologous proteins (Collawn *et al.*, 1991; Letourneur and Klausner, 1992). The primary sequence of ubiquitin carries two candidates for these signals: Tyr59, which is exposed on the surface of the molecule (Vijay-Kumar *et al.*, 1985); and DQQRLL, which is similar to the DKQTLL internalization signal that was defined in the CD3 subunit of the T-cell receptor (Letourneur and Klausner, 1992) (Figure 6A). Mutation of Tyr59 to alanine had no effect on the ability of ubiquitin to promote internalization of α -factor by Ste2p-376-Ub (see Figure 7C). A sequence related to the tyrosine-based NPXY signal, NPFXD, has been shown to function as a transferable internalization signal in yeast (Tan *et al.*, 1996). Ubiquitin does not contain an NPFXD sequence although it has two Phe residues on its surface, one of which is important for endocytosis (see below).

The potential di-leucine motif in ubiquitin actually has the sequence LI, not LL, but LI has been shown to substitute for LL (Letourneur and Klausner, 1992; Pieters *et al.*, 1993; Odorizzi, 1994; Dietrich *et al.*, 1997). The leucine in DQQRLL, Leu43, is a hydrophobic residue buried in the interior of the folded ubiquitin structure (Vijay-Kumar *et al.*, 1985) and therefore mutation of this residue to alanine is likely to disrupt proper folding of the polypeptide (see below). Because critical residues in this putative signal are buried within the folded polypeptide, it is unlikely that DQQRLL is recognized by the endocytic machinery. Nevertheless, to rule out the possibility that DQQRLL is a di-leucine signal in ubiquitin, we tested whether DQQRLL could restore internalization of a version of Ste2p that was truncated at amino acid 340 and lacked its single ubiquitylation site (see Figure 1). It has been shown for the CD3 di-leucine signal that the transfer of six amino acids, DKQTLL, is sufficient to induce the internalization of a heterologous protein (Letourneur and Klausner, 1992). However, DQQRLL did not rescue internalization of pheromone by the truncated receptor, although fusion of the entire ubiquitin polypeptide did (Figure 6B). Thus, DQQRLL does not act as a di-leucine internalization signal, and ubiquitin does not carry one of the classic internalization signals that have been defined previously.

To determine whether ubiquitin carries a linear internalization signal, or whether the three-dimensional folded structure of the ubiquitin polypeptide is necessary for promoting receptor internalization, we made mutations in Ste2p-376-Ub that were expected to disrupt folding of the ubiquitin moiety. Figure 6C shows the rapid internalization of Ste2p-376-Ub3xR, in which Lys29, Lys48 and Lys63 of ubiquitin have been mutated to Arg to prevent all polyubiquitin chain formation (Arnason and Ellison, 1994). The truncated receptor that lacks all post-translational ubiquitylation sites and was not fused to ubiquitin (Ste2p-4xR,378Stop) was internalized 4.3-fold more slowly (Table I). This represents the ubiquitin-independent background level of internalization for this experiment. Mutation of ubiquitin Val26, a buried residue in the three-dimensional ubiquitin structure, to Gly severely destabilizes ubiquitin by retarding folding kinetics (Khorasanizadeh *et al.*, 1996). In addition, UbV26G can not serve as a sole source of ubiquitin for a yeast cell (unpublished data), consistent with an important role for this amino acid in the structure of the protein. The

A

M Q I F V K T L T G K T I T L E V E S S D T I D N V K S K I Q D K E G I P P
D Q Q R L I F A G K Q L E D G R T L S D Y N I Q K E S T L H L V L R L R G G

B **C**

Fig. 6. Ubiquitin internalization information is not a tyrosine- or di-leucine-based signal but is carried in the three-dimensional structure of the polypeptide. (A) Sequence of ubiquitin. Shown in bold and underlined are residues that may be part of well-characterized plasma membrane protein internalization motifs. (B and C) α -factor internalization assays were performed on cells expressing different Ste2p and Ste2p-Ub mutants (see Figure 1). Cells expressing these receptors were grown to log phase in SD minimal medium and assayed as described in Figure 2A. All curves represent the average of at least three independent experiments and error bars represent the standard deviations. (B) Ste2p-340-Ub (LHY713, □); Ste2p-337R,340Stop (LHY848, ◇); Ste2p-340-DQQRLI (LHY1518, ●). (C) Ste2p-376-Ub3xR (LHY1127, □); Ste2p-4xR,378Stop (LHY319, ◇); Ste2p-376-UbV26G (LHY1157, ■); Ste2p-376-UbL43A (LHY1162, ●).

mutation of Val26 to Gly in Ste2p-376-Ub3xR also inhibits internalization of α -factor by the chimeric protein (Figure 6C). To analyze the effect of mutating another buried hydrophobic residue that is likely to be required for protein stability we mutated Leu43 to alanine. Ubiquitin with the mutation Leu43 to Ala also can not serve as the sole source of ubiquitin in the cell (K.E.Sloper-Mould and L.Hicke, unpublished data) and the mutation abolishes the internalization of α -factor by Ste2p-376-Ub3xR (Figure 6C). The effect of mutating Val26 and Leu43 within the ubiquitin hydrophobic core indicates that a properly folded ubiquitin polypeptide is required to promote internalization.

The ubiquitin internalization signal is two surface patches bridged by a lysine residue

To define the internalization signal carried in the three-dimensional ubiquitin structure we performed scanning alanine mutagenesis of all the surface residues in the globular domain of ubiquitin. We mutated ubiquitin amino acids individually or in clusters of 2–4 residues in the Ste2p-376-Ub3xR chimeric protein. Most of these mutations had little or no effect on the internalization of α -factor by the chimera (for example see Figure 7C). However, the mutation of two amino acids markedly decreased the internalization rate of Ste2p-376-Ub3xR. The Ile44A and Phe4A mutations decreased the internalization rate 3.4- and 2.6-fold respectively, effects similar to those observed by disrupting the folded structure of ubiquitin (Figure 7A and B; Table I). We defined mutations

that internalized α -factor 1.5- to 2-fold more slowly than Ste2p-376-Ub3xR to have a partial defect in α -factor internalization. The mutations to alanine of five amino acids that lie near Phe4 or Ile44 have the most severe defects of mutations in this class. These mutations, Q2A, K6A, L8A, E64A and V70A, decreased internalization of Ste2p-Ub ~2-fold (Figure 7A and B; Table I). The mutations of other ubiquitin surface residues to alanine in the globular domain resulted in a <1.5-fold decrease in α -factor internalization rates (Figure 7C; K.E.Sloper-Mould and L.Hicke, unpublished data).

We mapped the amino acids that are required for internalization of Ste2p-Ub on the surface of the ubiquitin polypeptide (Figure 8). We observed that the residues important for internalization are localized to two surface patches that are bridged by Lys6. One of these patches consists of the hydrophobic residues Ile44, Leu8 and Val70. The other patch, Phe4, Gln2 and Glu64, is located on the same side of the ubiquitin molecule but is not contiguous with the first patch. The two patches are bridged by a protruding side chain belonging to Lys6. Mutation of Lys6 has a partial effect on the function of ubiquitin in internalization, whereas mutation of neighboring His68 has little if any effect (Figure 7C). Thus, two surface residues in the globular domain of ubiquitin are critical for its function as an internalization signal. These residues, Ile44 and Phe4, are found on distinct surfaces of the ubiquitin polypeptide and are part of patches formed with nearby residues that play a significant but lesser role in internalization.

Fig. 7. Mutations in ubiquitin surface residues that inhibit Ste2p-Ub internalization. α -factor internalization assays were performed on cells expressing different Ste2p-Ub mutant receptors. The Ste2p-Ub chimeras carried mutations of ubiquitin lysines 29, 48 and 63 to arginine. Cells expressing these receptors were grown to log phase in SD minimal medium and assayed as described in Figure 2A. All curves represent the average of at least three independent experiments and error bars represent the standard deviations. (A) Ste2p-376-Ub3xR (LHY1127, ◆); Ste2p-376-UbV26G (LHY1157, ◇); Ste2p-376-UbI44A (LHY1186, ■); Ste2p-376-UbL8A (LHY1340, ▲); Ste2p-376-UbV70A (LHY1338, ●); Ste2p-376-UbV26G (LHY1157, ◇); Ste2p-376-UbF4A (LHY1516, ■); Ste2p-376-UbQ2A (LHY1386, ▲); Ste2p-376-UbE64A (LHY1596, ●); Ste2p-376-UbK6A (LHY1676, ▼). (C) Ste2p-376-Ub3xR (LHY1127, ◆); Ste2p-376-UbV26G (LHY1157, ◇); Ste2p-376-UbY59A (LHY1153, ■); Ste2p-376-UbH68A (LHY1381, ●).

Discussion

At least two models exist to explain the mechanism by which ubiquitin triggers internalization of plasma membrane proteins. It has been suggested that ubi-

Table I. Effect of surface ubiquitin mutations on endocytosis

Mutation	Internalization half-time (min)	Relative inhibition of internalization
376-Ub3xR	11.4	1
4xR,378Stop	49.5	4.3
V26G	31.3	2.7
L43A	34.7	3.0
F4A	30.2	2.6
I44A	39.4	3.4
Q2A	17.8	1.6
K6A	19.4	1.7
L8A	21.0	1.8
E64A	17.9	1.6
V70A	17.9	1.6
Y59A	9.8	0.9
H68A	12.7	1.1

quitylation might promote the unfolding of linked proteins (Pickart, 1998); therefore, ubiquitin may expose a concealed internalization signal in endocytic cargo by inducing a structural change in the protein. Alternatively, ubiquitin itself may contain an internalization signal that is appended to a protein. Here we show that ubiquitin carries an endocytosis signal in its amino acid sequence that is sufficient to promote internalization of plasma membrane proteins. This signal is not one of the previously defined linear peptide internalization signals, but consists of patches on the surface of the folded ubiquitin polypeptide.

We have demonstrated that the fusion of ubiquitin in-frame to receptors that lack ubiquitin sites restores the ability of the receptors to be internalized. Endocytosis of the Ste2p-Ub chimeric proteins follows a pathway similar to that taken by wild-type Ste2p because internalization of Ste2p or Ste2p-376-Ub requires the same cellular machinery, including the Pan1, End3 and End4 proteins. The previously defined SINNDKSS internalization signal is not required for endocytosis of a receptor chimera that carries a ubiquitin moiety. Therefore, SINNDKSS serves as a strong ubiquitylation motif but does not in itself carry additional information important for receptor internalization. These results suggest that serine phosphorylation is necessary for internalization solely to promote ubiquitylation, although we have not formally demonstrated that the Ste2p-Ub proteins are not phosphorylated in the Gly/Ser linker or on the ubiquitin moiety.

Other ubiquitylation motifs that promote endocytosis have been defined in the cytoplasmic domains of the α -factor receptor, Ste3p, and a peptide transporter, Ste6p (Kölling and Losko, 1997; Roth *et al.*, 1998). A PEST-like sequence that is required for ubiquitylation of the uracil permease has also been identified (Marchal *et al.*, 1998). Like the SINNDKSS motif, these sequences are rich in serines, threonines and negatively charged amino acids, and include lysine residues. We propose that these motifs may all promote endocytosis by simply serving as sites for interaction with the ubiquitylation machinery, allowing a ubiquitin internalization signal to be appended.

Ubiquitin is able to promote internalization when fused to a severely truncated receptor that carries only six amino acids of its cytoplasmic tail (Ste2p-303-Ub), suggesting that ubiquitin carries information sufficient for internaliz-

Fig. 8. Internalization information carried by ubiquitin is present in two surface patches bridged by a lysine residue. The three-dimensional structure of ubiquitin (Vijay-Kumar *et al.*, 1985) is presented at two different angles in images constructed with RasMol molecular visualization software (Sayle and Milner-White, 1995). Residues essential for internalization are highlighted in magenta; surrounding residues that function in endocytosis but play a lesser role are shown in pink.

Table II. Yeast strains

Strain	Genotype
LHY10	<i>MATa ura3 ste2::LEU2 leu2 his3 trp1 bar1-1</i>
LHY18	<i>ura3::ste2-378Stop[URA3]</i> , same as LHY10
LHY319	<i>ura3::ste2-337R,352R,358R,374R,378Stop[URA3]</i> , same as LHY10
LHY558	<i>ura3::ste2-337R,352R,358R,374R,376-UBIK48R[URA3]</i> , same as LHY10
LHY713	<i>ura3::ste2-337R340-UBIK48R[URA3]</i> , same as LHY10
LHY844	<i>ura3::ste2-337R,340-UBIK48R[URA3]</i> , same as LHY10
LHY847	<i>ura3::ste2-340Stop[URA3]</i> , same as LHY10
LHY848	<i>ura3::ste2-337R,340Stop[URA3]</i> , same as LHY10
LHY852	<i>ura3::ste2-320Stop[URA3]</i> , same as LHY10
LHY1078	<i>ura3::ste2-312SINNDKSS[URA3]</i> , same as LHY10
LHY1127	<i>ura3::ste2-337R,352R,358R,374R376-Ub29R,48R,63R,[URA3]</i> , same as LHY10
LHY1153	<i>ura3::ste2-337R,352R,358R,374R376-Ub29R,48R,Y59A,63R[URA3]</i> , same as LHY10
LHY1157	<i>ura3::ste2-337R,352R,358R,374R376-UbV26G,29R,48R,63R[URA3]</i> , same as LHY10
LHY1162	<i>ura3::ste2-337R,352R,358R,374R-Ub29R,L43A,48R,63R[URA3]</i> , same as LHY10
LHY1186	<i>ura3::ste2-337R,352R,358R,374R-Ub29R,I44A,48R,63R[URA3]</i> , same as LHY10
LHY1194	<i>ura3::ste2-321-UBIK48R[URA3]</i> , same as LHY10
LHY1212	<i>ura3::ste2-303-UBIK48R[URA3]</i> , same as LHY10
LHY1338	<i>ura3::ste2-337R,352R,358R,374R-Ub29R,48R,63R,V70A[URA3]</i> , same as LHY10
LHY1340	<i>ura3::ste2-337R,352R,358R,374R-UbL8A,29R,48R,63R[URA3]</i> , same as LHY10
LHY1381	<i>ura3::ste2-337R,352R,358R,374R-Ub29R,48R,63R,H68A[URA3]</i> , same as LHY10
LHY1386	<i>ura3::ste2-337R,352R,358R,374R-UbQ2A,29R,48R,63R[URA3]</i> , same as LHY10
LHY1464	<i>pGAL-HA-PMA1[URA3] MATa ura3 leu2 his4 lys2 bar1-1</i>
LHY1465	<i>pGAL-HA-PMA1-UBIK48R[URA3] MATa ura3 leu2 his4 lys2 bar1-1</i>
LHY1469	<i>pGAL-HA-PMA1-UBIK48R[URA3] MATa pan1-20 ura3 leu2 trp1 lys2 his3 bar1-1</i>
LHY1471	<i>pGAL-HA-PMA1-UBIK48R[URA3] MATa ura3 leu2-3,112 his3-11,15</i>
LHY1474	<i>pGAL-HA-PMA1-UBIK48R[URA3] MATa pre1-1 pre2-2 ura3 leu2-3,112 his3-11,15</i>
LHY1477	<i>pGAL-HA-PMA1-UBIK48R[URA3] MATa ura3-1 leu2-3,112 his3-11 ade2-1 can1-100</i>
LHY1479	<i>pGAL-HA-PMA1-UBIK48R[URA3] MATa pep4::HIS3 prb1::LEU2 ura3-1 leu2-3,122 his3-11 ade2-1 can1-100</i>
LHY1487	<i>pGAL-HA-PMA1-UBIK48R[URA3] MATa sec6 his4 leu2 lys2 bar1</i>
LHY1488	<i>ura3::ste2-337R,352R,358R,374R,376-UBIK48R [URA3] MATa ste2::LEU2 end4-1 ura3 trp1 leu2 bar1 his3 and/or his4</i>
LHY1490	<i>ura3::ste2-337R,352R,358R,374R,376-UBIK48R[URA3] MATa ste2::LEU2 end3-1 ura3 leu2 his4 ade2 bar1</i>
LHY1516	<i>ura3::ste2-337R,352R,358R,374R-UbF4A,29R,48R,63R[URA3]</i> , same as LHY10
LHY1518	<i>ura3::ste2-337R,340-DQQLI[URA3]</i> , same as LHY10
LHY1580	<i>ura3::ste2-337R,352R,358R,374R,376-UBIK48R[URA3] MATa ste2::LEU2 pan1-20 ura3-52 his3-Δ200 trp1Δ901 leu2-3,112 lys2-801 suc2-Δ9 bar1::LYS2</i>
LHY1596	<i>ura3::ste2-337R,352R,358R,374R-Ub29R,48R,63R,E64A[URA3]</i> , same as LHY10
LHY1676	<i>ura3::ste2-337R,352R,358R,374R-UbK6A,29R,48R,63R[URA3]</i> , same as LHY10

ation. The SINNDKSS sequence is also able to promote internalization when fused to a receptor that lacks all internalization information (Ste2p-312–SINNDKSS)

(Rohrer *et al.*, 1993). Interestingly, the Ste2p-303–Ub chimera ($t_{1/2} = 57.6$ min) is internalized more rapidly than Ste2p-312–SINNDKSS ($t_{1/2} = 76$ min), as is Ste2p-340–

Ub ($t_{1/2} = 12.8$ min) compared with Ste2p-340Stop ($t_{1/2} = 28.3$ min). These observations suggest that ubiquitylation is rate limiting for internalization, at least for truncated versions of the receptor. More evidence that ubiquitin is sufficient for internalization comes from the observation that the fusion of ubiquitin is enough to induce the internalization of a truncated receptor that lacks ubiquitin sites even in the absence of pheromone.

Ubiquitin is not only necessary and sufficient for Ste2p internalization, but can serve as a transferable internalization signal. It was shown previously that large sequences carrying ubiquitylation motifs from Ste3p and Ste6p could be added to the stable plasma membrane ATPase to promote rapid degradation of this protein (Kölling and Losko, 1997; Roth *et al.*, 1998). We have now shown that fusion of only ubiquitin itself to the C-terminus of Pma1p significantly increased turnover of this protein. The increase in degradation induced by ubiquitin was due to increased internalization and subsequent degradation of this protein in the vacuole. Degradation of Pma1p-Ub was prevented in a mutant that blocks the fusion of secretory vesicles with the plasma membrane, in mutants that can not endocytose, and in a mutant that lacks active vacuolar hydrolases. In contrast, mutations that inhibit proteasome activity did not affect Pma1p-Ub degradation, indicating that the proteasome is not involved. Thus, Ste2p-Ub and Pma1p-Ub travel the same endocytic pathway as ubiquitylated wild-type Ste2p and are ultimately degraded in the vacuole. Ubiquitin by itself can function as an internalization signal.

Our results do not rule out the possibility that the optimal function of ubiquitin in promoting internalization is context dependent. On the contrary, we found that the distance of ubiquitin from the plasma membrane influences how efficiently it triggers endocytosis. The internalization of Ste2p-303-Ub ($t_{1/2} = 57.6$ min) is less efficient than internalization of Ste2p-321-Ub ($t_{1/2} = 18.5$ min) or Ste2p-340-Ub ($t_{1/2} = 12.8$ min). Perhaps ubiquitin is more accessible to interacting proteins when it is further from the membrane. The orientation of ubiquitin with respect to the membrane and other endocytic cargo may also affect its efficacy in promoting internalization. Finally, some ubiquitylated plasma membrane proteins such as Ste3p, Gap1p and the EGF receptor also carry Tyr/Phe-based or di-leucine internalization sequences (Chang *et al.*, 1993; Hein and André, 1997; Roth *et al.*, 1998). How these internalization signals function together with ubiquitin remains to be determined.

What is the internalization information carried by ubiquitin? Ubiquitin has one tyrosine residue and a DQQRLLI sequence that may act as traditional tyrosine- or di-leucine-based signals. However, we have shown that neither of these established linear signals function to promote endocytosis. Instead, we find that the ubiquitin signal requires three-dimensional structure, since destabilizing the ubiquitin folded structure inactivates the signal. The ubiquitin structure consists of a single globular domain made up of a five-stranded β -sheet and an α -helix. Protruding from this domain is a flexible 4-amino-acid C-terminal tail (Vijay-Kumar *et al.*, 1985). The structure has a dense hydrophobic core and several surface hydrophobic patches. We have found that two patches on the surface of the ubiquitin globular domain carry information

necessary for endocytosis of a receptor-ubiquitin chimera; we have not yet determined the role of the ubiquitin tail in endocytosis. Two surface residues, Ile44 and Phe4, were absolutely required for internalization because their mutation to alanine affected Ste2p-Ub internalization as severely as mutations that destabilized the ubiquitin structure. Scanning alanine mutagenesis of other ubiquitin surface residues revealed that the majority of surface amino acids play little or no role in internalization. In most of the ubiquitin protein, even the simultaneous mutation of 3–4 consecutive amino acids had little effect on internalization. Although most of the ubiquitin surface residues are not involved in endocytosis, they do play a significant role in other cellular functions because most mutants could not serve as a sole source of ubiquitin for a yeast cell (K.E.Sloper-Mould and L.Hicke, unpublished data).

Ile44 is part of a hydrophobic surface patch that includes Leu8 and Val70. The residues in this patch are important for the degradation of ubiquitylated proteins by the proteasome (Beal *et al.*, 1996). Leu8 and Val70 play a role in endocytosis, although they are not as important as Ile44. Pickart and colleagues have demonstrated that mutations in these three hydrophobic residues result in mutant ubiquitin molecules that are able to conjugate relatively efficiently to substrate proteins, indicating that the mutant proteins can be recognized by the ubiquitylation machinery and have a reasonably stable structure (Beal *et al.*, 1996). Therefore, the effect of the I44A, L8A and V70A mutations on endocytosis is unlikely to be due to unstable structure of the mutant proteins. Phe4 is the critical residue in another patch on the surface of ubiquitin important for endocytosis. This patch also includes Gln2 and Glu64. It is possible that mutations in Phe4 destabilize the ubiquitin protein; however, the side chain of this residue is completely exposed on the ubiquitin surface (see Figure 8). Furthermore, mutation of Phe4 to Tyr, a conservative substitution that is not expected to disrupt structure, has as severe an effect on endocytosis as mutation to Ala (K.E.Sloper-Mould and L.Hicke, unpublished data). Therefore, we believe that mutation of Phe4 might abrogate endocytosis not by disturbing ubiquitin structure, but by disrupting a protein-protein interaction that promotes ubiquitin-dependent internalization.

In mammalian cells, tyrosine- and di-leucine-based internalization signals promote endocytosis by interacting with adaptors (AP complexes) and clathrin. Yeast AP complexes play little or no role in Ste2p internalization (Huang *et al.*, 1999). In addition, internalization of the EGF receptor, which is ubiquitylated, does not require the AP2 adaptor protein (Nesterov *et al.*, 1999). Therefore, ubiquitin may promote internalization by linking internalized proteins to the endocytic machinery via a specialized type of adaptor. Such a mechanism promotes the internalization of the β -adrenergic receptor. Upon activation, this receptor becomes phosphorylated on serine residues; phosphorylated serines then provide binding sites for the protein arrestin, a specialized adaptor that links activated receptor to the clathrin-based endocytic machinery (Goodman *et al.*, 1996). Our finding that monoubiquitin, in the absence of receptor tail sequences, is sufficient for internalization suggests that an adaptor protein that recognizes monoubiquitin would need to be sequestered

away from the free ubiquitin in the cell that might compete with ubiquitylated plasma membrane proteins for binding. This might be accomplished by localization of the adaptor to specific regions of the plasma membrane. Alternatively, an adaptor that recognizes ubiquitin may require a multivalent ubiquitin interaction that occurs non-covalently upon multimerization of ubiquitylated receptors. Perhaps one of the patches important for internalization promotes clustering of endocytic cargo proteins and the other provides a binding site for interacting proteins. Alternatively, the Ile44 and Phe4 patches, bridged by Lys6, could provide binding sites for one or more plasma membrane proteins that would interact with endocytic cargo to promote efficient internalization.

Materials and methods

Strains, plasmids, media and reagents

Yeast strains are listed in Table II. The plasmids containing *STE2* variants were transformed into *ste2Δ* strains by single-step gene transplacement at the *ura3* locus. All mutant Ste2 proteins were able to restore mating and α -factor binding to the *ste2Δ* parental strain. The expression of Ste2p mutant proteins was assessed by immunoblotting using anti-Ste2p polyclonal antibodies. Each mutant Ste2p migrated at the expected molecular weight. Two individual transformants of each mutant were assayed for their ability to internalize α -factor, and in each case both transformants demonstrated similar internalization kinetics.

All mutations in the *STE2* sequence were constructed by site-directed mutagenesis using the two-step polymerase chain reaction (PCR) method (Higuchi *et al.*, 1988). DNA sequences of the mutagenized PCR fragments and in-frame ligation junctions were confirmed by automated sequencing. To introduce an in-frame ubiquitin fusion, a unique *Bam*HI site was created at different positions of *STE2*. This was followed by an in-frame ligation of a PCR-amplified DNA fragment encoding a glycine-rich linker (GGGSGGGTGGGSGGG; Robinson and Sauer, 1996) and UbK48R flanked by *Bam*HI restriction sites. The *Bam*HI site introduced at the codon for amino acid 320 resulted in three amino acid changes: F319L, Y320D and G321P. These residues were mutated back to the original sequence by site-directed mutagenesis resulting in LHP583. To fuse ubiquitin at amino acid 303, a unique *Nhe*I site was introduced at amino acid 302 followed by in-frame ligation of DNA encoding glycine-linked UbK48R as described above (LHP611).

Plasmid LHP719 was constructed by amplification of a PCR fragment from pJR3 (Rohrer *et al.*, 1993) using a primer that introduced a *Bam*HI site at the codon for amino acid 340, followed by the sequence encoding DQQRLL-Stop. This PCR product was ligated into a plasmid carrying a *Bam*HI site introduced into *STE2* at amino acid 340.

Plasmid LHP609 containing *GAL1-HA-PMA1-UBI* was derived from pRK315, which contains a unique *Bam*HI site behind the last codon of *GAL1-HA-PMA1* (Kölling and Losko, 1997). The ~250 bp *Bam*HI fragment containing glycine-linked ubiquitin from LHP412 was cloned into the *Bam*HI site of pRK315 and the sequence of the *PMA1-UBI* junction was confirmed.

All strains were propagated in synthetic minimal (SD) medium (Sherman, 1991), casamino acid medium lacking uracil (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.5% vitamin assay casamino acids from Difco, supplemented with 50 mg/l adenine, histidine, tryptophan and methionine) or rich (YPUAD) medium (2% bacto peptone, 1% yeast extract, 2% glucose). The purification of 35 S-labeled α -factor has been described previously (Singer and Riezman, 1990; Dulic *et al.*, 1991). Ste2p polyclonal antibody was purified as described (Hicke and Riezman, 1996). The HA 12CA5 monoclonal antibody was a kind gift from Dr Robert A.Lamb (Northwestern University).

Ubiquitin mutagenesis

All Ste2p-376-Ub ubiquitin mutants were constructed by site-directed mutagenesis of template plasmid LHP587 using specific primers designed to introduce the required mutations (Q2A, F4A, K6A, L8A, V26G, L43A, I44A, Y59A, E64A, H68A or V70A). LHP587 (Ste2p-376-Ub3xR) was constructed in two steps. Initially, the *Kpn*I sites in LHP361 were deleted and the *Bam*HI site at the 3' end of *UBI* was mutated to *Kpn*I. Using the resulting plasmid (LHP372) as a template, Lys29 and Lys63 in ubiquitin were mutated to arginines with mutagenic primers

and a QuickChange™ Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All mutations were confirmed by automated sequencing.

Receptor clearance and α -factor internalization assays

All internalization assays were performed as described previously (Dulic *et al.*, 1991; Terrell *et al.*, 1998). For each assay, the growth and assay conditions are indicated in the figure legend. Internalization half-times were calculated based on an exponential curve-fit performed with KaleidaGraph software (Synergy Software, Reading, PA).

Receptor clearance assays were performed as described (Hicke *et al.*, 1998). Briefly, cells were treated with 10 μ g/ μ l cycloheximide for 20 min at 30°C to inhibit new protein synthesis. At various times aliquots of cells were transferred into buffer containing 10 mM Na₃N and 10 mM NaF. To measure ligand-stimulated receptor clearance, 1×10^{-6} M synthetic α -factor was added prior to removing the 0 min time point aliquot. Synthetic α -factor was then dissociated from poisoned cells by shaking the cells for 3 h at 30°C. The number of receptors that remained on the cell surface at each time point was determined by incubating cells with 35 S-labeled α -factor at a saturating concentration.

Cell lysates and immunoblots

To express Pma1p or Pma1p-Ub from the *GAL1* promoter, cells were grown to log phase at the appropriate temperature in casamino acid medium containing 2% galactose and 1% raffinose. Cells were harvested and resuspended to 5×10^6 cells/ml in casamino acid medium containing 2% glucose to inhibit Pma1p or Pma1p-Ub synthesis. After cells were allowed to recover at 30°C, or were shifted to the restrictive temperature for 20 min, a 10 ml aliquot was removed from the culture and resuspended in 20 mM (final concentration) NaF/Na₃N and placed on ice. This represented the 0 h chase time point. After 2, 3 and 4 h, 10 ml aliquots were removed and treated as described above.

The cells were collected by centrifugation at 4°C and washed once in 1 ml TEPI, 5 mM NEM (Terrell *et al.*, 1998) in 2.2 ml microcentrifuge tubes. Cells were resuspended in 100 μ l TEPI, ~0.2 g of glass beads were added, and cells were lysed by vortexing for 4 min at maximum speed. The cell lysate was diluted with 100 μ l of urea, 5% SDS buffer (Terrell *et al.*, 1998) and heated to 37°C for 10 min. The diluted lysate was removed and transferred to a 1.5 ml tube, and centrifuged at 4°C for 10 min at 20 800 g to remove cell debris. Bromophenol Blue (0.0002%) and β -mercaptoethanol (2%) were added to the cell lysate and proteins were resolved by 6% SDS-PAGE and transferred to Immobilon membranes (Millipore Corp., Bedford, MA). Membranes were blocked with 5% non-fat dried milk, 0.1% NP-40, 50 mM Tris-HCl pH 7.5, 0.15 M NaCl, followed by incubation with 12CA5 monoclonal HA antibody at 1:5000 dilution. Goat anti-mouse immunoglobulin conjugated with horseradish peroxidase (Sigma, St Louis, MO) was used at 1:5000 dilution and the immunoblot was developed with SuperSignal reagents (Pierce, Rockford, IL). To quantify the degradation of Pma1p and Pma1p-Ub, goat anti-mouse IgG + IgM alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as secondary antibody and visualized using ECF substrate (Amersham Pharmacia Biotech, Piscataway, NJ). The intensity of each protein signal was determined using Molecular Dynamics Storm 860 ImageQuant 1.11 software (Sunnyvale, CA). Degradation half-times were determined from a linear curve fit using Microsoft Excel software (Microsoft Corporation).

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