Multiple Roles for Rsp5p-dependent Ubiquitination at the Internalization Step of Endocytosis*

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Ubiquitination of integral plasma membrane proteins triggers their rapid internalization into the endocytic pathway. The yeast ubiquitin ligase Rsp5p, a homologue of mammalian Nedd4 and Itch, is required for the ubiquitination and subsequent internalization of multiple plasma membrane proteins, including the α-factor receptor (Ste2p). Here we demonstrate that Rsp5p plays multiple roles at the internalization step of endocytosis. Temperature-sensitive rsp5 mutant cells were defective in the internalization of α-factor by a Ste2p-ubiquitin chimera, a receptor that does not require post-translational ubiquitination. Similarly, a modified version of Ste2p bearing a NPFXD linear peptide sequence as its only internalization signal was not internalized in rsp5 cells. Internalization of these variant receptors was dependent on the catalytic cysteine residue of Rsp5p and on ubiquitin-conjugating enzymes that bind Rsp5p. Thus, a Rsp5p-dependent ubiquitination event is required for internalization mediated by ubiquitin-dependent and -independent endocytosis signals. Constitutive Ste2p-ubiquitin internalization and fluid-phase endocytosis also required active ubiquitination machinery, including Rsp5p. These observations indicate that Rsp5p-dependent ubiquitination of a trans-acting protein component of the endocytosis machinery is required for the internalization step of endocytosis.

Ubiquitin is a highly conserved 76-amino acid polypeptide that becomes covalently linked to substrate proteins by an isopeptide bond. Two characterized functions of protein ubiquitination are to target proteins for degradation by the cytosolic 26 S proteasome or to promote the internalization of cell surface proteins into the endocytic pathway. Recognition of cytosolic proteins by the proteasome generally requires modification with a polyubiquitin chain of at least four ubiquitin subunits (1, 2). In contrast, modification of plasma membrane proteins with monoubiquitin or Lys63-linked di-ubiquitin chains triggers internalization into the endocytic pathway (3–8), ultimately leading to degradation in the lumen of the lysosome.

Protein ubiquitination is catalyzed by a cascade of three enzymes (reviewed in Refs. 9 and 10). Ubiquitin-activating enzymes (E1s) activate ubiquitin in an ATP-dependent reaction, forming a high-energy thiolester bond with ubiquitin. The activated ubiquitin is then passed to a cysteine residue in a ubiquitin-conjugating enzyme (E2). Normally, E2s function together with ubiquitin ligases (E3s) to catalyze isopeptide bond formation between the carboxyl terminus of ubiquitin and ε-amino groups in lysine side chains of specific substrates. E3s bind directly to substrates and comprise the major specificity determinants of the ubiquitination machinery. There are two major classes of E3s. One class carries a RING finger domain, and the other carries a hect (homologous to E6-AP carboxyl terminus) domain. RING finger E3s function as adapter proteins, bringing the substrate to the ubiquitin-charged E2, whereas E3s of the hect domain family participate directly in catalysis by forming a thiolester with ubiquitin during the ubiquitination reaction.

Ubiquitin-dependent endocytosis regulates the cell surface activities of diverse plasma membrane proteins (reviewed in Refs. 11 and 12). In yeast, ubiquitin is a widely used endocytosis signal that promotes the internalization of plasma membrane proteins such as peptide pheromone receptors, transporters, and nutrient permeases. In mammalian cells, ubiquitin and the ubiquitination machinery regulate the endocytosis of the growth hormone receptor, epithelial sodium channel, epidermal growth factor receptor, and colony-stimulating factor receptor (13–16). In addition, many more mammalian signal-transducing receptors are known to undergo ligand-stimulated ubiquitination (reviewed in Ref. 17).

The mammalian hect domain E3s Nedd4 and Itch recognize and ubiquitinate the epithelial sodium channel and Notch, respectively (14, 18, 20). Rsp5p, a homologue of Nedd4 and Itch, promotes the regulated ubiquitination of plasma membrane proteins in yeast (reviewed in Ref. 21). Rsp5p is an essential protein that is implicated in a number of cellular processes in addition to its role in endocytosis. Rsp5p carries an amino-terminal C2 domain, three WW protein-protein interaction domains, and a carboxyl-terminal hect catalytic domain. Structure-function analyses have indicated that the WW domains of Rsp5p play an important role in its endocytotic function (22, 23). All three WW domains are required for normal rates of stimulated internalization of the α-factor receptor Ste2p and the uracil permease Fur4p, implicating these domains in the recognition of endocytic substrates (22, 23). The role of the C2 domain in endocytosis is less clear. Deletion of this domain has

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1 The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; EGF, epidermal growth factor; GHR, growth hormone receptor; HA, hemagglutinin epitope; YPUAD medium, rich medium; SD medium, synthetic minimal medium; LY, Lucifer Yellow; Ub, ubiquitin; PCR, polymerase chain reaction.
no effect on Ste2p internalization, but causes an increase in Gap1p and Fur4p activities at the cell surface and delayed fluid-phase endocytic transport to the vacuole (22, 24, 25). Thus, the C2 domain may be required for the internalization of a subset of plasma membrane proteins and/or may be involved in sorting within the endocytic pathway.

Observations in both yeast and mammalian cells have suggested that the role of ubiquitin in endocytosis may extend beyond its function as an appended internalization signal. The UbE motif, an endocytosis signal in the growth hormone receptor (GHR), is required for GHR internalization by a ubiquitin/proteasome-dependent pathway (26, 27). Because UbE-dependent internalization does not require ubiquitination sites in the receptor, Govers et al. (26) suggested that the binding of the ubiquitination machinery to the UbE motif, not ubiquitination of the GHR itself, is the critical event for internalization. Association of the ubiquitination machinery with the receptor may be required to recruit endocytic proteins to the GHR, or the ubiquitination machinery may modify a receptor-associated regulatory protein (26). Endocytic proteins are known to be substrates of the ubiquitin system. Eps15, an essential component of the clathrin-based endocytic machinery, undergoes epidermal growth factor (EGF)-stimulated monoubiquitination in fibroblasts (28). Genetic studies in Drosophila have suggested that cell fate specification in the compound eye requires specific deubiquitination of Liquid facets, the Drosophila orthologue of the endocytic protein epsin (29). In yeast, a rsp5 mutant lacking an intact C2 domain could ubiquitinate the Gap1 amino acid permease normally but was defective in permease down-regulation (24). This observation suggests that the requirement for Rsp5p in endocytosis cannot be fully explained by its role in modifying specific cargo proteins.

As a model for the function of ubiquitin and ubiquitin ligases in the down-regulation of plasma membrane proteins, we have studied the role of Rsp5p in the internalization of the yeast α-factor receptor, Ste2p. Ste2p is a MATα cell-specific G-protein-coupled receptor that initiates an intracellular signal required for yeast mating in response to α-factor binding (re- viewed in Ref. 30). Ligand binding also induces the sequential hyperphosphorylation and ubiquitination of the cytoplasmic tail of the receptor, modifications that trigger rapid receptor internalization (31, 32). After internalization, receptors are delivered to the lysosome-like vacuole, where they are permanently inactivated by degradation (33). Mutation of lysines in the receptor cytoplasmic domain or mutations that impair Rsp5p or the UbC1pUbC4pUbC5p E2s disrupt the ubiquitination and internalization of Ste2p (22, 31). Monoubiquitination by Rsp5p is sufficient to induce Ste2p internalization, since fusion of a single ubiquitin moiety to the cytoplasmic domain rescues the defective internalization of a receptor lacking post-translational ubiquitination sites (4). The internalization information in ubiquitin has been mapped to two distinct hydrophobic patches on the surface of the ubiquitin molecule (34); however, the mechanism by which ubiquitin induces internalization is undefined.

In this study, we used α-factor receptor variants that do not require post-translational ubiquitin modification to uncover a novel function of Rsp5p in endocytosis that is distinct from its role in ubiquitination of plasma membrane cargo. We found that the Rsp5p catalytic cysteine, ubiquitin-conjugating enzymes, and free cellular ubiquitin were required for internalization mediated by ubiquitin-dependent and -independent endocytosis signals. Our results indicate that Rsp5p has two distinct roles at the internalization step of endocytosis: the regulated ubiquitination of cargo proteins and the ubiquitination of a component of the constitutive endocytic machinery. This study demonstrates a requirement for trans-acting ubiquitination in endocytosis and identifies Rsp5p and Ubc1p/Ubc4p/Ubc5p as the enzymatic machinery required for this event.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Reagents—** The genotypes of strains used in this study are listed in Table I. Cells were propagated in synthetic minimal (SD) medium (35), or rich (YPUD) medium (2% bactopeptone, 1% yeast extract, 2% glucose, supplemented with 20 mg/ml adenine, methionine, and tryptophan). The purification of His6-Labeled α-factor and Ste2p antimiotin was performed as described previously (36, 37). Hemagglutinin (HA 12CA5) monoclonal antibodies were provided by Robert Lamb (Northwestern University, Evanston, IL). The ras8-2 mutation was provided by Jentsch and colleagues (38). The ras8-1 allele was isolated by Winston and colleagues (Harvard Medical School, Boston, MA) and was characterized by Wang et al. (39).

**Plasmid Construction—** All mutations in the STE2 sequence were constructed by site-directed mutagenesis using the two-step polymerase chain reaction (PCR) procedure (40). To generate the ste2-2NPFxD mutant, the mutation G392N was introduced into a mutant ste2 (ste2-7xR) with Lys to Arg mutations at amino acids 337, 352, 358, 374, 387, 400, and 422 (4). To generate ste2-All Lys, the F394A mutation was introduced into pMR1 (41) by PCR mutagenesis. Sequences encoding ste2p-NPFxD and Ste2p-All Lys were subsequently subcloned into the centromeric plasmid YCP1a3 (42) resulting in LHP426 and LHP427, respectively. Construction of ste2-ubi (LHP361) and ste2-378Stop (LHP147) has been described previously (4). The ste2-ubi construct used in this study contains a single ubiquitin fused to the amino acid sequence derived from GHR, or mutations that impair endocytosis and identifies Rsp5p and Ubc1p/Ubc4p/Ubc5p as the enzymatic machinery required for this event. All ste2 variants were able to complement the mating defect of ste2Δ cells. RSP5 plasmids were based on the yeast-Asche-

**α-Factor Receptor and Lucifer Yellow Endocytosis Assays—** α-Factor internalization assays were performed as described (22, 37). Specific variations in growth conditions and assays are indicated in the figure legends. Cells were grown to early logarithmic phase, harvested and centrifuged, and suspended in YPUD medium at 5 × 10⁵ cells/ml. For continuous presence assays, cells were shifted to the nonpermissive temperature for 15 min prior to the addition of 7S-labeled α-factor. For pulse-chase internalization assays, 35S-labeled α-factor was bound to cells on ice for 45–60 min. Unbound radioactivity was removed by centrifugation at 4 °C, and internalization was initiated by the addition of media warmed to 30 °C or 37 °C as indicated in the figure legends. The internalization of internalization is expressed as the ratio of internalized to total cell-associated radioactivity. For unknown reasons, ∆ubc1A ∆ubc4A expressed a low level of α-factor binding. Loss of Ubc1p and Ubc4p has also been reported to cause a decrease in maltose permease expression (44). For this reason, internalization assays depicted in Fig. 5A were corrected by subtracting nonspecific cell-associated radioactivity that bound to ste2Δ cells under the same conditions. This correction did not significantly alter the results of the assay but allowed a more accurate measurement of initial internalization rates.

Receptor clearance assays were performed as described (32) with the following changes: Cells were shifted to 37 °C at the same time as the addition of cycloheximide to 10 µg/ml. The 0-min time point was taken 30 min after cycloheximide treatment was initiated to allow Ste2p en route through the biosynthetic pathway to reach the cell surface. The number of α-factor receptor binding sites present on the cell surface at each time point was determined by binding with a mix of 35S-labeled α-factor and ∼3 × 10⁶ µM unlabeled a-factor to ensure that a-factor binding sites were saturated in each sample.

Lucifer Yellow (LY) endocytosis assays were performed as described previously (22, 37). As indicated in the figure legends, cells were shifted to 37 °C for 15 min prior to the addition of 10 µl of 40 mg/ml Lucifer Yellow Citrate (Fluka Chemika-Biochemika, Switzerland), or LY was immediately added at 30 °C. After 60 min of incubation, the assay was stopped by the addition of 1 ml of ice-cold phosphate/stop buffer (50 mM sodium phosphate, pH 7.5, 10 mM sodium azide, 10 mM sodium fluoride). Cells were viewed using a Zeiss LSM410 confocal microscope equipped with fluorescein isothiocyanate filter sets.

**Cell Lysates and Immunoblots—** Lysates for Ste2p immunoblotting were prepared as described previously (31) with minor modifications.
Cells were grown in SD medium to early logarithmic phase, harvested by centrifugation, and transferred to YPUD medium. Cells were incubated 12.5 min at 37 °C before treatment with 1 × 10 −6 M α-factor for 8 min. Cells were not treated with cycloheximide. Lysis was achieved by mechanical agitation with glass beads in urea/SDS buffer containing 5 mM N-ethylmaleimide to inhibit isopeptidase activities in the extract. Lysates were supplemented with β-mercaptoethanol (2%) and bromphenol blue (0.002%) and incubated at 37 °C for 10–15 min prior to loading. Extracts used for detecting HA-Rsp5p in Fig. 4A were prepared by an alkaline lysis protocol as described (45). Immunoblotting was performed as described previously (22).

**RESULTS**

**Rsp5p Is Required for Internalization of Receptors That Do Not Require Post-translational Ubiquitination—Ste2p-Ub is a chimeric, functional α-factor receptor that lacks post-translational ubiquitination sites but carries a monoubiquitin moiety fused in-frame to the cytosol-terminal cytoplasmic tail (Fig. 1). We have shown previously that the fused ubiquitin moiety is sufficient to signal internalization (4, 34). Because the internalization of Ste2p-Ub does not require post-translational ubiquitin conjugation, we hypothesized that Ste2p-Ub internalization would not depend on Rsp5p. To test this idea, we compared the internalization of Ste2p-Ub with a receptor that carries the same tail sequence but lacks a fused ubiquitin and requires post-translational ubiquitination for internalization (Ste2p-378Stop, see Fig. 1). The ability of these receptors to internalize α-factor was examined in the rasp−2 temperature-sensitive mutant and in wild-type cells (Fig. 2). Both Ste2p-Ub and Ste2p-378Stop were internalized rapidly in wild-type cells. Consistent with previous observations, rasp−2 cells were unable to internalize Ste2p-378Stop. The internalization of Ste2p-Ub in rasp−2 cells was also defective, although it occurred more rapidly than the internalization of Ste2p-378Stop. These data suggest that fusion of ubiquitin to the receptor partially relieves the requirement for Rsp5p in receptor internalization, consistent with its role in appending ubiquitin to Ste2p.

**Table I**

| Strain        | Genotype                                                                 |
|---------------|---------------------------------------------------------------------------|
| LHY18         | MATa ura3::ste2–378Stop::URA3 ste2::LEU2 ura3 leu2 his3 trp1 bar1          |
| LHY180        | MATa ubc1::HIS3 ura3 leu2 his3 trp1 bar1                                  |
| LHY196        | MATa ubc4::HIS3 ubc5::LEU2 ura3 leu2 his3 his4 lys2 trp1                   |
| LHY558        | MATa ura3::ste2–ubiK48R::URA3 ste2::LEU2 ura3 leu2 his3 trp1 bar1         |
| LHY559        | MATa ura3::ste2–378Stop::URA3 ras5::HIS3 leu2::ras5–2::LEU2 ste2::LEU2 lys2 trp1 his3 bar1 |
| LHY560        | MATa ura3::ste2–ubiK48R::URA3 ras5::HIS3 leu2::ras5–2::LEU2 lys2 trp1 his3 bar1 |
| LHY883        | MATa ras5–1 leu2 ura3 bar1                                               |
| LHY886        | MATa leu2 ura3 his4 trp1 bar1                                             |
| LHY757        | MATa pste2-NPFXD::URA3 ras5::HIS3 leu2::ras5–2::LEU2 ste2::LEU2 lys2 trp1 his3 bar1 |
| LHY758        | MATa pste2-NPFXD::URA3 ste2::LEU2 leu2 trp1 his3 ura3                    |
| LHY759        | MATa pste2::All Lys[URA3] ras5::HIS3 leu2::ras5–2::LEU2 ste2::LEU2 lys2 trp1 his3 bar1 |
| LHY760        | MATa pste2::All Lys[URA3] ste2::LEU2 lys2 trp1 his3 ura3                  |
| LHY1042       | MATa pste2-NPFXD::URA3 ste2::LEU2 ras5–1 ura3 leu2 trp1 bar1              |
| LHY1044       | MATa pste2::All Lys[URA3] ste2::LEU2 ras5–1 ura3 leu2 trp1 bar1           |
| LHY1122       | MATa ura3::ste2–ubiK48R::URA3 ras5–1 ste2::LEU2 ura3 leu2 trp1 bar1       |
| LHY1160       | pHA-RSP5[TRP1], same as LHY1042                                          |
| LHY1161       | pHA-rsp5C777A[TRP1], same as LHY1044                                     |
| LHY1192       | pHA-RSP5[TRP1], same as LHY1044                                          |
| LHY1193       | pHA-rsp5C777A[TRP1], same as LHY1044                                     |
| LHY1194       | MATa ubc1::HIS3 ubc4::HIS3 ura3 leu2 trp1 lys2 his3 bar1                  |
| LHY1198       | MATa ura3::ste2–ubiK48R::URA3 ste2::TRP1 his3 trp1 lys2 ura3 leu2 bar1    |
| LHY1502       | MATa ura3::ste2–378Stop::URA3 ste2::TRP1 his3 trp1 lys2 ura3 leu2 bar1   |
| LHY1503       | MATa ura3::ste2–378Stop::URA3 ste2::TRP1 his3 trp1 lys2 ura3 leu2 bar1   |
| LHY1506       | MATa ura3::ste2–ubiK48R::URA3 ste2::TRP1 his3 trp1 lys2 ura3 leu2 bar1   |
| LHY1507       | MATa ura3::ste2–378Stop::URA3 ste2::TRP1 his3 trp1 lys2 ura3 leu2 bar1   |
| LHY1510       | MATa ura3::ste2–378Stop::URA3 ste2::LEU2 his3 ade2 ura3 leu2 trp1 lys2 bar1 |
| LHY1511       | MATa ura3::ste2–378Stop::URA3 ste2::LEU2 his3 ade2 ura3 leu2 trp1 lys2 bar1 |
| LHY1562       | MATa pSTE2[URA3] end4–1 ste2::LEU2 ura3 trp1 leu2 bar1 his3 and/or his4  |
| LHY1634       | pHA-RSP5[TRP1], same as LHY1122                                          |
| LHY1635       | pHA-rsp5C777A[TRP1], same as LHY1122                                     |
| LHY1955       | MATa pste2-NPFXD::URA3 ste2::LEU2 ura3 trp1 leu2 bar1 his3 and/or his4    |

**Fig. 1.** Schematic representation of Ste2p cytoplasmic tail variants. The positions of tail lysines and lysine → arginine mutations are indicated. The bold arrow indicates mutations in the NPFXD-like internalization signal. TM, seventh (last) transmembrane domain of Ste2p. (g), Gly/Ser linker. UBI, fused ubiquitin moiety.

Activated receptors, but that Rsp5p is also required downstream of receptor modification.

Two distinct classes of internalization signals function in yeast. In addition to ubiquitin, a linear aromatic residue-based sequence, NPFXD, that is present in the cytosolic domain of the α-factor receptor, Ste3p, can act as an efficient internalization signal. A weak version of the NPFXD signal, GPFAD, is present in the cytosol-tail of wild-type Ste2p. To analyze receptor endocytosis that is mediated solely by the NPFXD signal, we generated a mutant receptor with all tail ubiquitination sites mutated to Arg and with the mutation G392N to convert the GPFAD sequence to a strong NPFXD signal (Ste2p-NPFXD, Fig. 1). 2 Ste2p-NPFXD is internalized with kinetics similar to wild-type Ste2p (see Fig. 3B). To confirm that Ste2p-

2 J. Howard and G. Payne, personal communication.
Rsp5p is required for the internalization of a Ste2p-ubiquitin chimera. Plasmids encoding Ste2p-Ub and Ste2p-378Stop were introduced into rps5–2 ste2A and RSP5 ste2Δ cells. Continuous presence α-factor internalization assays were performed at 37 °C. Cells were grown in YPUAD medium at 24 °C and pre-incubated at 37 °C for 15 min prior to the addition of radiolabeled α-factor. Figure shows RSP5 Ste2p-378Stop (LHY18, ■), rps5–2 Ste2p-378Stop (LHY559, □), RSP5 Ste2p-Ub (LHY558, ●), and rps5–2 Ste2p-Ub (LHY560, ○). Curves represent the average of at least three independent assays, and error bars indicate the standard deviation at each time point.

NPFXD does not undergo ligand-stimulated ubiquitination at the cell surface, we expressed Ste2p-NPFXD and wild-type Ste2p in an end4–1 mutant. The end4–1 mutation causes a complete block in the internalization step of endocytosis at 37 °C (47), and activated receptors accumulate as phosphorylated and ubiquitinated forms at the plasma membrane (31). Ste2p-NPFXD was efficiently hyperphosphorylated, but did not undergo detectable levels of ubiquitination, whereas wild-type Ste2p was ubiquitinated in the presence of ligand (Fig. 3A). As a control for internalization assays, we constructed a Ste2p variant that bears only ubiquitin-dependent internalization signals by mutating Phe-394 to Ala to inactivate the endogenous GPfad signal (Ste2p-All Lys, see Fig. 1). When we measured α-factor internalization in rps5–2 and wild-type strains expressing these receptors, we found that Ste2p-NPFXD and Ste2p-All Lys were equally affected by the rps5 mutation (Fig. 3B). These observations indicate that Rsp5p is required for internalization mediated by both known classes of yeast internalization signals, even when the ubiquitination of endocytic cargo is not necessary. Thus, Rsp5p is required for a novel function in endocytosis downstream of cargo modification.

Ubiquitination of a Non-receptor Substrate Is Required for Rapid Receptor Internalization—The internalization defect of Ste2p-Ub and Ste2p-NPFXD in rps5 cells could be explained in two different ways. First, Rsp5p could function to ubiquitinate a trans-acting protein that functions in endocytosis. Alternatively, the amino-terminal domains of Rsp5p could participate in a non-catalytic function that is essential for endocytosis. To distinguish between these possibilities, we investigated whether catalytically inactive Rsp5p carrying a mutation in the Nterminal domain could promote internalization of Ste2p-Ub and Ste2p-NPFXD. The C777A mutation abolishes thiolester formation with ubiquitin (48), and this mutant cannot serve as the sole source of Rsp5p in the cell (24, 39). We constructed an (HA) epitope-tagged version of Rsp5p that is fully functional (22) and a similarly tagged mutant version with the C777A mutation.

The C777A mutation did not affect Rsp5p expression (39, Fig. 4A). To compare the function of wild-type Rsp5p and Rsp5pC777A in endocytosis, we expressed Rsp5p and Rsp5pC777A in rps5–1 cells and assayed α-factor internalization after inactivating the endogenous Rsp5–1p by incubation at 37 °C. We used the rps5–1 allele for this set of experiments because expression of Rsp5pC777A in rps5–2 cells caused a dramatic growth defect even at the normally permissive temperature of 24 °C. The endocytosis defect of rps5–1 cells was only partially rescued by plasmid-borne wild-type Rsp5p (compare Fig. 4 (B–D) with Figs. 2 and 3B), because rps5–1 has a semidominant effect on α-factor internalization, perhaps due to the formation of mixed Rsp5–1p/Rsp5p multimers (22). Rsp5pC777A did not rescue the internalization of α-factor by Ste2p-All Lys, as expected since Ste2p-All Lys requires ubiquitination to be internalized (Fig. 4B). Rsp5pC777A was also unable to rescue Ste2p-Ub and Ste2p-NPFXD internalization in rps5–1 cells (Fig. 4, C and D), indicating that the catalytic function of Rsp5p is essential for internalization of receptors that do not require post-translational ubiquitin modification.

To confirm that a Rsp5p-dependent ubiquitination event was required for Ste2p-Ub and Ste2p-NPFXD internalization, we performed two additional experiments. First, we tested the role of E2 enzymes in the internalization of Ste2p-Ub. The E2 enzymes Ubc1p, Ubc4p, and Ubc5p are homologous and form an essential gene family (49). Wild-type Ste2p internalization is impaired in ubc1Δ ubc4Δ and ubc4Δ ubc6Δ mutants (31). To test whether the internalization of Ste2p-Ub depends on the function of these enzymes, we generated ubc1Δ ubc4Δ and congenic UBC1 UBC4 strains expressing Ste2p-Ub and Ste2p-378Stop. The internalization of both Ste2p-Ub and Ste2p-378Stop was slower in ubc1Δ ubc4Δ than in wild-type cells;
However, the internalization of Ste2p-Ub was slightly more rapid than that of the receptor requiring post-translational ubiquitination (Fig. 5A), similar to the internalization of Ste2p-Ub in rsp5 cells. Ubiquitin-conjugating enzymes were also required for Ste2p-NPFXDX internalization.3

Second, we tested whether the internalization of Ste2p-Ub was dependent on the levels of free ubiquitin in the cell. To do this we assayed Ste2p-Ub and Ste2p-378Stop internalization in a doa4Δ mutant. DOA4 encodes a deubiquitinating enzyme, and the pool of free ubiquitin is severely reduced in doa4Δ cells because conjugated ubiquitin is not recycled efficiently (50). The rate of Ste2p-Ub internalization was decreased in doa4Δ cells, although it was somewhat faster than the internalization of Ste2p-378Stop (Fig. 5B). Internalization of both Ste2p-378Stop and Ste2p-Ub in doa4Δ cells was restored by the overexpression of ubiquitin.4 These data indicate that Ste2p-Ub internalization depends on ubiquitin-conjugating enzymes and normal levels of free ubiquitin in the cell.

Ubiquitination Is Required for Constitutive Endocytosis—Components of the endocytic machinery can be modified in a signal-dependent manner. One example of this is the EGF-induced tyrosine phosphorylation of clathrin heavy chain, an event that influences clathrin localization and EGF receptor endocytosis (51). It has also been suggested that Ca2+-triggered dephosphorylation of endocytic proteins may represent a general mechanism to stimulate the assembly of endocytic coats after nerve terminal depolarization (52). Ste2p is constitutively ubiquitinated at a low level, and receptor ubiquitination can mediate the slow constitutive internalization that occurs in the absence of ligand (32). If the novel function of Rsp5p in endocytosis involved the ligand-stimulated modification of a protein involved in endocytosis, then the constitutive internalization of Ste2p-Ub would be unaffected by rsp5 mutations. To determine whether Rsp5p plays a role in constitutive internalization downstream of receptor ubiquitination, we measured the clearance of Ste2p-Ub from the surface of cells in the absence of α-factor. The constitutive internalization of Ste2p-Ub was dramatically impaired in rsp5Δ cells as compared with the internalization of Ste2p-All Lys.
pared with wild-type cells (Fig. 6). Therefore, Rsp5p-dependent ubiquitination of a trans-acting endocytic protein is not exclusively coupled to receptor activation.

Because the involvement of Rsp5p seemed to be a constitutive, signal-independent requirement, we investigated the role of ubiquitination in fluid phase endocytosis. LY is a soluble fluorescent molecule that is internalized by fluid-phase endocytosis and delivered to the vacuole (37). Mutants that block the internalization step of endocytosis cannot localize LY to the vacuole (37). Previous studies showed that Rsp5p and a subset of its amino-terminal domains are required for LY localization to the vacuole (22, 23, 53). We tested whether the role of Rsp5p in fluid phase endocytosis involves protein ubiquitination by analyzing fluid-phase endocytosis in mutants deficient in ubiquitin-conjugating enzymes that cooperate with Rsp5p. We performed LY endocytosis assays in ubc1Δ ubc4Δ and ubc4Δ ubc5Δ cells and compared these mutants with wild-type cells and cells deficient in only ubc1Δ, which transport LY normally. ubc1Δ ubc4Δ cells showed a decrease in vacuolar fluorescence intensity, and ubc4Δ ubc5Δ cells showed an even stronger defect compared with ubc1Δ cells (Fig. 7). Consistent with previous studies, rps5–1 cells were also unable to internalize LY efficiently (Fig. 7). These data support the conclusion that Rsp5p-dependent ubiquitin ligation to a trans-acting protein is required for efficient constitutive internalization from the plasma membrane.

DISCUSSION

In this study, we demonstrate that the Rsp5 ubiquitin ligase is required to regulate an unknown component of the endocytic machinery by ubiquitination. Previously, Rsp5p has been shown to modify endocytic cargo with a three-dimensional ubiquitin internalization signal (reviewed in Ref. 21). A second function for Rsp5p-dependent ubiquitination in endocytosis was revealed in our experiments using receptors that do not require cis-acting, post-translational modification by ubiquitin for internalization. We found that the internalization of a chimeric α-factor receptor carrying a functional ubiquitin internalization signal was dependent on Rsp5p catalytic activity, ubiquitin-conjugating enzymes, and on ubiquitin itself. These data argue strongly that receptor internalization requires Rsp5p-mediated ubiquitination of an unidentified protein. Rsp5p-dependent ubiquitination was also required for internalization mediated by a Ste2p variant bearing the linear peptide internalization signal NPFXD, for fluid-phase endocytosis, and for constitutive internalization of the Ste2p-ubiquitin chimera.

Therefore, the novel Rsp5p-dependent function is not exclusively coupled to ligand-stimulation or to internalization mediated by the ubiquitin internalization signal, but instead is a constitutive requirement for the internalization step of endocytosis. We propose that Rsp5p regulates one or more constitutive components of the endocytosis machinery by ubiquitination.

An alternative explanation for our observations is that Rsp5p is required for the internalization of Ste2p-Ub and Ste2p-NPFXD because a threshold level of cargo ubiquitination by Rsp5p is essential for the productive formation of primary endocytic vesicles. However, we consider this explanation unlikely for the following reasons. First, we found that the expression of an abundant, internalized plasma membrane protein carrying the ubiquitin signal, Pma1p-Ub (34), did not influence the internalization of Ste2p-Ub in rps5 mutant cells. Even though Pma1p-Ub was expressed at a high level, so that it represented 10–20% of the total plasma membrane protein in yeast (54), rps5 cells were still unable to internalize Ste2p-Ub. Second, the cytosolic domains of Sgl1p, Sro4p/Bud10p, and many other predicted plasma membrane proteins contain a perfect consensus NPFXD sequence. Since multiple plasma membrane proteins probably use the NPFXD internalization signal, a significant level of cargo competent for internalization may be available even in the absence of ubiquitin-modified cargo.

Recently, Jentsch and colleagues (38) described an essential role for Rsp5p in a novel activation pathway required for the synthesis of unsaturated fatty acids. The lethality of a rps5Δ mutation can be suppressed by overexpression of OLE1, a gene required for synthesis of oleic acid, or by exogenous addition of oleic acid to the growth medium. Oleic acid does not rescue the internalization of wild-type Ste2p in rps5 cells. This result was expected because Rsp5p is required to ubiquitinate Ste2p prior to internalization. We considered the possibility that the defective internalization of the Ste2p-Ub and receptors carrying the NPFXD signal could be due to a general oleic acid requirement for efficient endocytosis. However, we found that the exogenous addition of oleic acid to rps5 mutant cells carrying Ste2p-Ub...
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could not restore α-factor internalization, although the growth defect of rps5 cells was substantially rescued by oleic acid.3 Thus, our results cannot be explained by a deficiency in unsaturated fatty acids in rps5 mutant cells.

Several observations made by other investigators support the conclusion that the role of ubiquitin in endocytosis is not limited to tagging cargo proteins. Genetic evidence has suggested that cell fate specification in the Drosophila eye requires modulation of endocytosis through specific deubiquitination of the endocytic protein epsin (29). In mammalian cells, Eps15, an epsin-binding protein required for clathrin-mediated endocytosis, undergoes EGF-induced monoubiquitination (28). Finally, internalization of a mutant growth hormone receptor lacking ubiquitination sites is blocked by specific inhibitors of the proteasome or by temperature inactivation of a thermolabile E1 activity, suggesting the receptor itself does not require ubiquitination (26, 27).

What endocytic proteins might be regulated by Rsp5p? Yeast homologues of the EH domain protein Eps15 and the epsins are candidates because there is evidence for ubiquitin modification of their counterparts in different organisms. Furthermore, mutations in RSP5 and the Eps15 homologue PAN1 interact genetically (53). It has been proposed that Rsp5p and Pan1p interact via the binding of Rsp5p WW domains to proline-rich motifs in the carboxyl terminus of Pan1p (55). Rsp5p also contains a NPF motif, the ligand for EH domains found in Pan1p (56, 57). However, we have been unable to detect a physical interaction between Rsp5p and Pan1p, and we have not detected ubiquitinated forms of Pan1p or the yeast epsins Ent1p and Ent2p. In addition, disruption of the NPF sequence in Rsp5p has no affect on α-factor internalization.3 Chang et al. (58) showed that Rsp5p WW domains are type I WW domains that bind preferentially to PPXY-containing ligands. The yeast amphiphysin homologue, Rvs167p, and Arc15p, a component of the Arp2/3p actin complex that is required for endocytosis, contain PPXY motifs that may interact with Rsp5p WW domains. Thus, Arc15p and Rvs167p are candidate endocytic proteins that may be regulated by Rsp5p-mediated ubiquitination. Since some WW domains have affinity for phosphoserine and phosphothreonine (59), the WW domains of Rsp5p may interact with and promote ubiquitination of endocytic phosphoproteins. Because interactions that require post-translational modification cannot be easily predicted by sequence gazing, the identification of Rsp5p-regulated endocytic proteins may require a combination of unbiased genetic and biochemical approaches.

A rps5 mutant lacking its C2 domain is competent for nitrogen-regulated ubiquitination of the Gap1 permease but not for its subsequent degradation (24). This observation prompted the proposition that the C2 domain is required for interaction with or ubiquitination of an endocytic protein downstream of peroxisome ubiquitination (21, 60). We have observed that a mutant lacking the Rsp5p C2 domain exhibits wild-type internalization of α-factor by Ste2p-Ub or by Ste2p-NPF-XD,4 indicating that the C2 domain is not involved in the novel function of Rsp5p described in this study. Furthermore, our results indicate that the C2 domain is dispensable for internalization mediated by either of the defined endocytosis signals in yeast. We have, however, observed a defect in the transport of a fluid phase marker to the vacuole in a rps5-DΔC2 mutant (22), consistent with a role for the C2 domain in a post-internalization step of endocytosis. Our observations do not rule out the possibility that the C2 domain is required specifically for permease internalization.

What is the molecular mechanism by which Rsp5p regulates the constitutive endocytic machinery? The internalization of Ste2p-375Stop and Ste2p-Ub was not significantly affected by mutations in the PRE1 and PRE2 genes encoding catalytic subunits of the proteasome. Therefore, the novel function of Rsp5p-dependent ubiquitination is not likely to involve proteasomal degradation of a substrate protein. We propose that Rsp5p modulates the activity of the endocytic machinery by reversible modification with ubiquitin in a manner independent of the proteasome. There are a growing number of proteins whose modification by ubiquitin has been shown to regulate the activity of the protein without degradation. The core nuclear histones H2A and H2B are stably modified by ubiquitin in vivo (61), and loss of ubiquitination of H2B causes a meiosis defect in yeast (62). Monoubiquitination of the human protein FANC D2, a protein involved in ionizing radiation-induced DNA repair, causes its recruitment to nuclear foci containing other DNA repair factors including BRCA1 (63). Lys-63-linked ubiquitin chains modify the stable ribosomal protein L23 and activate the IεkB kinase TRAF6 (64, 65). Finally, ubiquitination negatively regulates the transcription factor Met4p, but ubiquitin-dependent inactivation of Met4p is clearly not accompanied by its proteasome-mediated degradation (19). We suggest that reversible ubiquitination of endocytic proteins acts to control protein activity. Modification with ubiquitin may alter protein activity by inducing a conformational change, changing the protein’s localization, or altering the affinity of the substrate for its protein or lipid binding partners. Molecular characterization of this function awaits the identification of the Rsp5p endocytic substrate(s).

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