Ubiquitination of the PEST-like Endocytosis Signal of the Yeast a-Factor Receptor*

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A 58-residue-long, PEST-like sequence within the yeast a-factor receptor (Ste3p) specifies the ubiquitination, endocytosis, and consequent vacuolar degradation of the receptor protein (Roth, A. F., Sullivan, D. M., and Davis, N. G. (1998) J. Cell Biol. 142, 949–961). The present work investigates three lysyl residues that map within this sequence as the potential ubiquitin acceptor sites. Lys → Arg substitution mutants were tested for effects on both ubiquitination and endocytosis. Results indicate that the three lysines function redundantly; a severe blockade to both ubiquitination and endocytosis is seen only for receptors having all three lysines replaced. Of the three, Lys432 plays the predominant role; ubiquitination and turnover are significantly impaired for receptors having just the K432R mutation. CNBr fragmentation of the receptor protein, used for the physical mapping of the ubiquitin attachment sites, showed PEST-like sequence lysines to be modified both with single ubiquitin moieties as well with short multi-ubiquitin chains, two or three ubiquitins long. Thus, in addition to being the signal for ubiquitination, the Ste3p PEST-like sequence also provides the site for ubiquitin attachment. To test if this endocytosis signal functions solely for ubiquitination, we have asked if the requirement for the PEST-like sequence in endocytosis might be bypassed through pre-attachment of ubiquitin to the receptor protein. Indeed, Ste3-ubiquitin translational fusions that have a ubiquitin moiety fused to the receptor in place of the PEST-like signal do undergo rapid endocytosis and vacuolar turnover. We conclude that ubiquitin alone, with no required contribution from receptor sequences, provides the sufficient signal for initiating uptake. In addition, our results confirm conclusions originally drawn from studies with the a-factor receptor (Terrell, J., Shih, S., Dunn, R., and Hicke, L. (1998) Mol. Cell 1, 193–202), namely that mono-ubiquitin, and not multi-ubiquitin chains provide the primary recognition determinant for uptake. Although mono-ubiquitination suffices, our results indicate that multi-ubiquitination serves to augment the rate of uptake.

The mechanisms that select plasma membrane substrates for clathrin-mediated endocytosis in mammalian cells are reasonably well understood (3, 4). Substrates subject to constitutive uptake display short peptidyl endocytosis signals centered around either an aromatic residue, usually a tyrosine, or a Leu-Leu dipeptide (5). These signals are decoded through the binding of the AP-2 adaptin complex which then serves to coordinate the assembly of the clathrin coat protein complex, thereby initiating the invagination of a patch of the plasma membrane into the cytoplasm. A variation on this theme has recently been described for the agonist-dependent uptake of the β2-adrenergic receptor (6). In this case, the adaptin role in clathrin-coated pit assembly is replaced by the binding of β-arrestin to the phosphorylated cytoplasmic tail domain of the liganded receptor. β-Arrestin also binds strongly to clathrin, thus allowing the assembly of the receptor into the clathrin-coated pit.

Endocytosis in the yeast Saccharomyces cerevisiae differs in several regards from the classic clathrin-dependent endocytosis of mammalian cells. While yeast express obvious homologues of the key players for clathrin-mediated uptake, i.e. clathrin heavy and light chains and the adaptin subunits, a clear demonstration of the requirement of these proteins in yeast uptake has remained elusive. Exhaustive deletion of the genes encoding the adaptin subunits is without discernible effect on yeast uptake processes (7). For clathrin, while mutations that disable clathrin function show partial endocytosis defects, the interpretation of this partial defect remains uncertain (8, 9). Clathrin may either function redundantly as one of several coat proteins functioning in yeast uptake, or alternatively, as these clathrin mutants also severely impair viability, the effect on endocytosis could be indirect.

In yeast, it is now clear for a variety of endocytic substrates that the selectivity of uptake depends at least in part on selective ubiquitination (10, 11). Plasma membrane proteins destined for uptake are marked with added ubiquitin. Nonetheless, as with the clathrin-mediated uptake, the selectivity of the process ultimately depends upon the recognition of determinants or signals encoded within the endocytic substrate’s amino acid sequence. Sequences involved in directing uptake have been identified for several yeast endocytic substrates (1, 12–15). Given the differences between clathrin- and ubiquitin-dependent uptake processes, it is not surprising that these yeast sequences bear little resemblance to the signals directing the clathrin-mediated endocytosis of mammalian cells. The two yeast pheromone receptors, the a-factor receptor (Ste2p) and the a-factor receptor (Ste3p), both have been well studied in terms of endocytosis. These two G protein-coupled receptors mediate the hormonal cross-talk that precedes the sexual conjugation of the MATa cell with the MATa cell. The two receptors are subject both to a ligand-dependent internalization mechanism as well as to a ligand-independent (i.e. constitutive) uptake mechanism. For Ste2p, the sequence SINNDAKSS, which maps to the regulatory C-terminal cytoplasmic tail do-
main (CTD), has been shown to be required for α-factor-induced uptake (12, 16). Mutations in this sequence block both ubiquitination and endocytosis. Indeed, the Lys of this sequence has been suggested to be the major site for ubiquitination as its replacement by Arg blocks both the ubiquitination and internalization of the receptor (16). The sequence NPF-STD, located within the Ste3p CTD, has been shown to be required for its α-factor-induced endocytosis (13). While required for uptake, these sequences within Ste2p and Ste3p clearly do not constitute sufficient signals for endocytosis. Additional receptor domains also are required. For instance, ligand-dependent uptake obviously also depends upon the ability of the receptor to bind ligand. Indeed, for Ste2p, in addition to the CTD sequence SINNDAKSS, mutations that map to predicted extracellular domains of the receptor also are found to impair ligand-dependent uptake (17).

One well characterized yeast endocytosis signal is the signal that directs the rapid constitutive, ligand-independent internalization of the α-factor receptor (1). For Ste3p, constitutive endocytosis is the more prominent of the two uptake modes. In the absence of a α-factor ligand, Ste3p is rapidly internalized from the cell surface and delivered to the vacuole for degradation (18). Ste3p consequently is a short-lived protein with a t1/2 of only 15 min in cells growing at 30 °C. This rapid endocytosis depends upon a 58 residue-long sequence mapping to the C terminus of the receptor CTD (1). Receptor mutants deleted for this sequence fail to undergo both constitutive ubiquitination and endocytosis and instead stably accumulate at the cell surface. Furthermore, this sequence also is a sufficient signal; when transplanted to the cytoplasmically disposed C terminus of the plasma membrane ATPase Pma1p, normally a stable cell surface resident, the Ste3p sequence directs both ubiquitination and rapid internalization of the ATPase to the vacuole for degradation. With its preponderance of both the acidic residues Asp and Glu and hydroxylated residues Ser and Thr, the Ste3p signal most closely resembles a class of ubiquitination signals known as PEST sequences (19). Similar sequences rich in acidic and hydroxylated residues have also been implicated in the endocytosis of both the α-factor export protein Ste6p and the uracil permease Fur4p (14, 15).

The present work extends the analysis of the Ste3p PEST-like endocytosis signal, particularly with regards to its role in ubiquitination. First, we ask if, in addition to specifying ubiquitination, the PEST-like sequence also provides the locus for ubiquitin attachment. Lys → Arg replacement mutagenesis of the three lysine residues located within the PEST-like sequence indicates that the three likely function redundantly as the sites for ubiquitin attachment: receptors having these three lysines replaced, fail to be ubiquitinated and fail to be internalized. Direct mapping of the ubiquitination site confirms the PEST-like signal as the primary locus for ubiquitin attachment. In addition, this analysis identifies the presence of short multi-ubiquitin chains attached therein. To test if these multi-ubiquitin chains might be required for directing uptake, we have applied the ubiquitin fusion methodology of Terrell et al. (2). Results with Ste3-ubiquitin fusions confirm results made previously with the Ste2-ubiquitin fusions (2), namely that recognition of mono-ubiquitin, and not the multi-ubiquitin chain, is key for endocytosis. In addition, the conclusions of Terrell et al. (2) are also extended with the demonstration that ubiquitin addition to a plasma membrane protein is not only necessary for initiating uptake, but also is sufficient; ubiquitin functions alone to specify uptake, not in conjunction with other receptor sequences or signals.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid pSL1904 serves as the starting point for many of STE3 mutations constructed in this work. pSL1904 is GAL1-STE3 carried on the URA3 integrating plasmid pRS306 (20). The presence of chromosomal flanking sequences upstream and downstream of the GAL1-STE3, 1.1 and 2.3 kilobase pairs long, respectively, allow both wild-type and mutant GAL1-STE3 constructs to be introduced back into yeast chromosome by homologous recombination in place of ste3Δ::LEU2 alleles. pSL1904 is identical to pSL1839 (1), except in having a 706-base pair GAL1,10 fragment promoter substituting for the STE3 promoter (21), replacing the sequences 417 to 110 base pairs upstream of the STE3 ORF.

The plasmid pND751 is identical to the CUP1-driven, myc-ubiquitin 2μ/URA3 expression plasmid pND747 (1), except that the Met 15 codon for the ubiquitin Met 15 codon (corresponding to ubiquitin Met 1) has been changed to a Leu codon. The M15L mutation removes a CNBr cleavage site that would otherwise result in the loss of the identifying myc epitope extension from the tagged ubiquitin. The M15L mutation was introduced by oligonucleotide mutagenesis (Table I) of single-stranded DNA derived from pND153, a BamHI to KpnI subclone of the 0.6-kilobase pair CUP1-myc-Ubi of pEP105 (22) into pRS306 (20). Finally, pND751 was derived by swapping the BamHI-KpnI fragment carrying the M15L mutation back into pND747.

Construction of Ste3-Ub Fusions—The ubiquitin sequences were joined to STE3 sequences at one of two XhoI sites previously introduced into STE3, one at codons 398/399 and the other at codons 466/467 (1). The Ste3Δ399-UB class of fusions lacks the 72 C-terminal residues of Ste3p (residues 399–470), including the PEST-like endocytosis signal that directs the rapid constitutive, ligand-independent uptake (18). The Ste3Δ399-UB fusions lack the PEST-like endocytosis signal and fail to undergo both constitutive ubiquitination and endocytosis and instead stably accumulate at the cell surface. Furthermore, this sequence also is a sufficient signal; when transplanted to the cytoplasmically disposed C terminus of the plasma membrane ATPase Pma1p, normally a stable cell surface resident, the Ste3p sequence directs both ubiquitination and rapid internalization of the ATPase to the vacuole for degradation. With its preponderance of both the acidic residues Asp and Glu and hydroxylated residues Ser and Thr, the Ste3p signal most closely resembles a class of ubiquitination signals known as PEST sequences (19). Similar sequences rich in acidic and hydroxylated residues have also been implicated in the endocytosis of both the α-factor export protein Ste6p and the uracil permease Fur4p (14, 15).

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The abbreviations used are: CTD, cytoplasmic tail domain; Ub, ubiquitin; PAGE, polyacrylamide gel electrophoresis.

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| Mutation          | Mutagenic oligonucleotide | Single-stranded DNA template | Restriction site* |
|-------------------|----------------------------|-------------------------------|------------------|
| GAL1-STE3(K424R)  | CGAGAGTAACTCCTGAGAAATCTCC  | pSL1904                      | XhoI             |
| GAL1-STE3(K432R)  | GAATTTCTCTTCTCTAGAAGCTGGAG  | pSL1904                      | Removes HindIII   |
| GAL1-STE3(K453R)  | GAATTCTCTTCTCTAGAAGCTGGAG  | pSL1904                      | XbaI             |
| GAL1-STE3(K424R,K432R) | GAATTTCTCTTCTCTAGAAGCTGGAG  | pSL1904 with K424R and K453R | Removes HindIII   |
| GAL1-STE3(K424R,K432R) | GAATTTCTCTTCTCTAGAAGCTGGAG  | pSL1904 with K424R and K453R | XbaI             |
| GAL1-STE3(K424R,K432R) | GAATTTCTCTTCTCTAGAAGCTGGAG  | pSL1904 with K424R and K453R | Neol             |
| GAL1-STE3(K424R,K453R) | GAATTTCTCTTCTCTAGAAGCTGGAG  | pSL1904 with K424R and K453R | SphI             |
| GAL1-STE3(K424R,K453R) | GAATTTCTCTTCTCTAGAAGCTGGAG  | pSL1904 with K424R and K453R | Neol             |
| GAL1-STE3(K432R)  | GAATTTCTCTTCTCTAGAAGCTGGAG  | pSL1904 with K424R and K453R | Neol             |
| GAL1-STE3(K424R,K432R) | GAATTTCTCTTCTCTAGAAGCTGGAG  | pSL1904 with K424R and K453R | Neol             |
| CUP1-myc-Ste3p    | GAATTTCTCTTCTCTAGAAGCTGGAG  | pND774                        | HaeII            |
| GAL1-STE3(339)-Ub(7K)    | CTAGTGGCATCCAGGCCTTCCTAGTC | pND774 with G76A             | Removes HaeII     |
| GAL1-STE3(339)-Ub(7K,R75-76) | GCATCTAGAGCTTCTGCTATGGAGA | pND774 with G76A             | Removes HaeII     |

* Each of the mutations either adds or removes a restriction enzyme digestion site, which was used diagnostically to identify plasmids with the mutations.

Assessment of Turnover and Ubiquitination—Ste3p and Ste3-Ub turnover was assessed via a non-radioactive pulse-chase protocol (18). A 2-h “pulse” of receptor synthesis was induced with the addition of 2% galactose to exponential cultures of cells carrying the GAL1-driven constructs growing in YP-raffinose (2%) medium. Following the “chase” period was instigated with the addition of 3% glucose. The analysis of Ste3-Ub protein turnover in some instances utilized a protocol in which cells were treated with energy poisons prior to the preparation of extracts. The conditions and the buffers utilized are identical to those used in the protease-shaving protocol, except that no protease is added.

Visualization of the ubiquitinated forms of the receptors was enhanced with treatment of the protein extracts with potato acid phosphatase (Roche Molecular Biochemicals) prior to analysis by SDS-PAGE and Western blotting (25).

Protease Digestion of Intact Cells—The treatment of whole cells with proteases (Promega Biotech) following centrifugation and protein extracts were prepared for SDS-PAGE and Western blotting as described previously (1). We have first focused on these three lysines as potential acceptor sites for the ubiquitination associated with constitutive endocytosis. Mutant receptors were constructed with conservative arginine substitutions at one, two, or all three lysines. Mutants were compared with wild-type Ste3p for effects on both turnover and ubiquitination.

Mutant receptors were expressed from the GAL1 promoter, and turnover was assessed by following the rate of receptor loss subsequent to glucose-mediated repression of new receptor synthesis. Turnover rates measured in this way are roughly equivalent to those measured via in vivo labeling, pulse-chase analysis of cells expressing Ste3p from its natural promoter (1, 18, 25). Analysis of the single lysyl replacements showed no detectable effect on turnover of either the K424R or K453R mutations (Fig. 2A). For K424R, however, turnover was clearly impaired, but not fully blocked. For receptors with two of the three lysines replaced (Fig. 2B), the K424R,K453R receptor displayed wild-type turnover kinetics. The other two double mutants, both of which included the K432R mutation, showed partial impairments similar to that observed for the single K432R mutant. The most complete blockade to turnover was seen for the triple 3K-R mutant (K424R,K432R,K453R), its turnover defect being substantially greater than any single or double mutants. Thus, while the K424R and K453R mutations alone or together showed essentially no perturbation of Ste3p turnover, it is evident from the 3K-R receptor results that these two residues clearly are capable of making at least a redundant contribution. Complete blockade of Ste3p turnover requires that all three of the lysines be substituted (Fig. 2B).

To assess receptor ubiquitination, mutant receptors were expressed in a pep4Δ cell context. As Ste3p turnover is blocked in pep4Δ cells, ubiquitinated receptor forms are not lost to...
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The Ste3p PEST-like endocytosis signal. The C-terminal 58 amino acid residues constituting the PEST-like endocytosis signal are shown with the three lysine residues indicated (boldface).

![Image](https://www.jbc.org/content/273/5/8146/F1.large.jpg)

**FIG. 1.** The Ste3p PEST-like endocytosis signal. The C-terminal 58 amino acid residues constituting the PEST-like endocytosis signal are shown with the three lysine residues indicated (boldface).

![Image](https://www.jbc.org/content/273/5/8146/F2.large.jpg)

**FIG. 2.** Turnover and ubiquitination of receptors having Lys → Arg mutations within the 58-residue PEST-like endocytosis signal. Strains were constructed in which GAL1-driven alleles of the three single mutants: Ste3(K424R)p, Ste3(K432R)p, and Ste3(K453R)p; the three double mutants: Ste3(K424R,K432R)p, Ste3(K424R,K453R)p, and Ste3(K432R,K453R)p; and the triple mutant Ste3(K424R,K432R,K453R)p (3K→R) were chromosomally integrated at the STE3 locus. Mutants were compared with wild-type Ste3p both for receptor turnover and ubiquitination. A, turnover of the single lysine → Arg mutants. NDT34-derived MATa strains were subjected to a 2-h period of galactose-induced receptor expression. Glucose was added to block further receptor synthesis, and, at the indicated times thereafter, culture aliquots were removed, extracts prepared, and the loss of Ste3 antigen was followed via SDS-PAGE and then Western blotting with Ste3p-specific antibodies. B, turnover of the three double lysine → Arg mutants. This experiment followed the protocol described for panel A. C, ubiquitination of the three single lysine → Arg mutants. Receptor expression was induced from pep4Δ versions (isogenic to NDT372) of the five strains utilized in panel A with a 2-h period of growth in galactose medium. Protein extracts prepared from these cells were treated with potato acid phosphatase and then subjected to SDS-PAGE and Western blotting with Ste3p-specific antibodies. D, ubiquitination of the three double lysine → Arg mutants. pep4Δ versions of the five strains utilized in panel B were cultured and treated as described above (panel C). Arrows at the left of panels C and D indicate the positions of the mono- and di-ubiquitinated forms of the receptor. The arrows at the right of panels C and D indicate the position of a protein that cross-reacts with the anti-Ste3p antibodies.

Vacuolar turnover. In addition, protein extracts were treated with phosphatase to remove heterogeneity in gel mobility due to phosphorylation of the receptor, thereby improving the Western blot visualization of minor ubiquitinated species (25). For the wild-type receptor, we find approximately 20% of isolated receptor is modified by ubiquitin, distributing equally between mono- and di-ubiquitinated forms (25). For the single Lys → Arg mutants (Fig. 2C), only K432R noticeably reduces ubiquitination levels. Likewise for the double mutants (Fig. 2D), only the two mutants that involve substitution of Lys432, i.e. K424R, K432R and K432R, K453R, reduce ubiquitination. The most severe reduction in receptor ubiquitination is seen for the 3K→R receptor (Fig. 2, C and D). As previously seen with deletions into the PEST-like sequence (1), receptor ubiquitination levels correlate well with turnover (Fig. 2, A and B).

While the 3K→R receptor shows the most complete ubiquitination impairment, a faint band is apparent at the monoubiquitinated receptor position, suggesting that the 3K→R receptor may remain subject to residual ubiquitination. Subsequent analyses confirm this low level mono-ubiquitination (Fig. 4).

The requirement for the PEST-like sequence lysines in receptor ubiquitination and turnover suggests that they serve as redundant acceptor sites for ubiquitin attachment. If so, a corollary conclusion would be that the 21 lysyl residues that map to other cytoplasmic portions of the receptor protein do not normally serve as acceptor sites. In addition, the significant impairments to ubiquitination and turnover caused solely by the K432R mutation, suggest a prominent role for this lysine; Lys432 may function as the primary ubiquitination site with Lys424 and Lys453 serving as alternative, secondary attachment sites.

The 3K→R Receptor Is Defective for the Internalization Step of Endocytosis—Evidence for a variety of yeast plasma membrane proteins indicates that ubiquitination acts at the cell surface internalization step of endocytosis (11). We expect, therefore, that the 3K→R receptor that lacks the presumptive ubiquitination sites is blocked for turnover because of failed internalization. If so, this receptor should accumulate at the plasma membrane. To assess receptor localization, we have used a protease-shaving protocol, wherein intact yeast cells are treated with proteases (18). Surface-localized receptor is degraded by the added proteases, while receptor residing intracellularly is unavailable for digestion. We have compared 3K→R receptor localization both to wild-type Ste3p and to the Δ413 truncation mutant, which is deleted for the PEST-like endocytosis/ubiquitination signal.

Ninety minutes after synthesis, wild-type Ste3p was found to be wholly resistant to the added, extracellular proteases (Fig. 3A), indicative of an intracellular localization and consistent with the vacuole as the known end point for Ste3p constitutive endocytosis (18). In contrast, Ste3Δ413p remains available to protease digestion, indicating a surface localization. The 3K→R mutant behaves essentially like Ste3Δ413p, remaining largely susceptible to the added proteases (>80% digested), indicating that the 3K→R accumulates at the plasma membrane, presumably a result of failed uptake.
In addition to showing the different localizations, the wild-type and the 3K→R receptors also manifest strikingly different electrophoretic presentations (Fig. 3); while the wild-type receptor appears as a heterogeneous cluster of bands, the 3K→R receptor migrates largely as a single, slightly lower molecular weight species (compare minus protease samples in Fig. 3). This difference in receptor modification status is explored more fully below (Fig. 6).

Determining the Site of Ubiquitination—For analysis of the Ste3p ubiquitination sites, we have made use of the K424R, K453R mutant (2K→R) receptor. This mutant, which retains Lys432, shows wild-type ubiquitination and turnover (Fig. 2D). If, as predicted by our mutational data, the PEST-like sequence lysines do serve as the primary ubiquitination sites, then 2K→R receptor ubiquitination should be largely limited to Lys432; this provides a simplification for our analyses.

The 185-residue-long Ste3p CTD contains only two methionines, Met288 and Met304, which map just to the C-terminal side of the seventh transmembrane domain (Fig. 5A). Cleavage of Ste3p with CNBr cleavage should release a 166-residue-long fragment extending from residue 305 to the C terminus at residue 470. As our Ste3p antibody was raised against the CTD, only this 305–470 fragment should be detected. If the sites for ubiquitin attachment are contained within this fragment, then ubiquitinated forms of the receptor should also be detected. For identification of the ubiquitinated receptor fragments, we have compared CNBr digests of the 2K→R receptor both to the 3K→R receptor (reduction in ubiquitinated species expected for the 3K→R receptor), and to 2K→R receptor isolated from cells overexpressing a myc-tagged ubiquitin (22). The epitope tag adds 14 residues to the N terminus of ubiquitin and Ste3p species modified with tagged ubiquitin rather than wild-type ubiquitin show gel migrations that are correspondingly slowed (25).

2K→R and 3K→R receptors were isolated from cells with wild-type ubiquitin expression levels or from cells that overexpress myc-ubiquitin. Intact receptors were analyzed first (Fig. 4A). 2K→R receptor isolated from the myc-ubiquitin-expressing cells showed modified receptor forms migrating at slightly higher molecular weights than the analogous receptor species modified with wild-type ubiquitin. In addition, receptor ubiquitination was greatly increased in cells expressing the myc-ubiquitin (the myc-ubiquitin construct is carried on a multicopy 2µ plasmid vector with expression being driven from the CUP1 promoter). Mono-, di-, and possibly tri-ubiquitinated species were apparent. 3K→R receptor ubiquitination remains impaired relative to 2K→R receptor even when isolated from the myc-ubiquitin overexpressing cells (Fig. 4A). Nonetheless, a species corresponding to mono-ubiquitinated 3K→R receptor is clearly evident, although di- and tri-ubiquitinated forms are not. Thus, the 3K→R receptor appears to be subject to a low level of mono-ubiquitination, which increases when ubiquitin is overproduced.

The protein extracts of Fig. 4A were subjected to CNBr cleavage and phosphatase digestion (Fig. 4B). For the 2K→R receptor extracted from the myc-ubiquitin cells, three prominent species show both increased intensity and retarded gel mobility relative to fragments from the control cell context (Fig. 4B). The three presumptive ubiquitinated species migrate near molecular weights calculated for the 305–470 CTD fragment with one, two, and three attached ubiquitin moieties, i.e. 28, 38, and 48 kDa. We conclude that the 305–470 receptor interval provides a locus for mono-, di-, and tri-ubiquitination. For the di- and tri-ubiquitinated fragments, this analysis does not distinguish if ubiquitins are attached as multi-ubiquitin chains or as mono-ubiquitins to separate lysine residues.

For 3K→R receptor, only the mono-ubiquitinated 305–470 fragment is apparent (Fig. 4B). As the 3K→R receptor lacks potential lysyl acceptor sites within the PEST-like sequence, this mono-ubiquitination must be attached to lysyl sites located elsewhere in the CTD.

Our expectation from the Lys→Arg mutational analysis was that most of 2K→R receptor ubiquitination should occur at Lys432. To test this, we have used site-directed mutagenesis to introduce methionyl sites for CNBr cleavage at various positions surrounding Lys432 (Fig. 5A). If Lys432 is a major site for ubiquitin attachment, then CNBr fragments that retain Lys432 should retain the ubiquitin modification, while fragments that do not retain Lys432 should not retain this modification (Fig. 5A). Methionines were introduced at sites within the PEST-like sequence expected to impact receptor ubiquitination and endocytosis minimally, based on previous mutagenic analysis of this sequence (1). Four receptor mutants were constructed with methionines introduced at residues 415, 421, 441, and 452 of the 2K→R receptor. The methionyl mutations were found to result in no obvious impairment to receptor endocytic function; turnover rates (data not shown) and ubiquitination levels (Fig. 5B) of the four were found to be roughly equivalent to that of the 2K→R parent.

Extracts from cells expressing the 2K→R receptor or the four mutant derivatives were subjected to CNBr digestion and then phosphatase treatment. In preliminary experiments, CNBr-generated CTD fragments from the five receptors showed different reactivities with the polyclonal anti-CTD antibody, presumably a reflection of the loss of epitopes as the CTD fragment is shortened from the C-terminal end through cleavage at the introduced methionines (Fig. 5A). To compensate for this, samples were normalized to give comparable detection of the major, 

![Figure 4](http://www.jbc.org/Downloadedfrom)
non-ubiquitinated CTD fragment (see Fig. 5C legend for details).

In Fig. 5C, we see that cleavage of the parental 2K→R receptor gives the major 305–470 fragment and the three higher molecular weight bands corresponding to this fragment with one, two, or three attached ubiquitins. Introduction of methionines into the 2K→R receptor results in predictable changes to the pattern of the CNBr peptide fragments. First, the major, non-ubiquitinated fragments (Fig. 5C, white dots) from the different methionyl mutants migrate predictably to positions consistent with the C-terminal shortening of the 305–470 fragment. (The one exception to this is the somewhat anomalous migration of the 305–421 fragment.) In addition, the corresponding ubiquitinated species also show the same downward gel mobility shift. Thus, the CNBr affinity clearing for receptors with cleavage sites at residues 441 and 452; the three ubiquitinated species show the predicted increases in gel mobility consistent with the C-terminal shortening of the 305–470 fragment (Fig. 5C, ubiquitinated species designated as 1, 2, and 3). Thus, the multi-ubiquitination seen for the 305–470 fragment is retained for both the 305–441 and 305–452 fragments. This contrasts sharply with results for receptors with methionines introduced at residues 413 and at 421. The 305–413 and 305–421 fragments clearly lack the the di- and tri-ubiquitinated species (the faint high molecular weight bands present in the Met413 and Met431 samples result from partial CNBr cleavage; described below). These fragments lack Lys432 and as they are clearly under-ubiquitinated, we conclude that Lys432 is indeed a major site for ubiquitin attachment. Furthermore, as the di- and tri-ubiquitinated species are lost simultaneously, we can conclude that Lys432 also provides a locus for the attachment of short multi-ubiquitin chains, two or three ubiquitin moieties long. For each of the methionine mutants, the identity of the ubiquitinated CTD fragments was confirmed as with the 2K→R receptor (Fig. 4B) through CNBr cleavage of extracts from cells overproducing the myc-tagged ubiquitin (data not shown).

Whereas the 305–413 and 305–421 fragments lack the di- and tri-ubiquitinated species, they do show evidence for some mono-ubiquitination. The amount of mono-ubiquitin present on these two species is lower than expected from Fig. 5C. The partial 289–470 cleavage product co-migrates at this position. Detectable levels of this partial fragment as well as the 305–470 partial fragment are present for each of four methionine mutants (Fig. 5C). Furthermore, to achieve equivalent antibody reactivity (see above), increased amounts of the Met413 and Met421 samples result from partial CNBr cleavage of the corresponding ubiquitinated species also show evidence for some mono-ubiquitination. These fragments lack Lys432 and as they are clearly under-ubiquitinated, we conclude that Lys432 is indeed a major site for ubiquitin attachment. Furthermore, as the di- and tri-ubiquitinated species are lost simultaneously, we can conclude that Lys432 also provides a locus for the attachment of short multi-ubiquitin chains, two or three ubiquitin moieties long. For each of the methionine mutants, the identity of the ubiquitinated CTD fragments was confirmed as with the 2K→R receptor (Fig. 4B) through CNBr cleavage of extracts from cells overproducing the myc-tagged ubiquitin (data not shown).
and Met\textsuperscript{421} samples were analyzed relative to the 2K→R parental sample. The 289–470 partial fragment that co-migrates with the mono-ubiquitinated 305–413 and 305–421 fragments is consequently over-represented in these samples, leading to an overestimate of ubiquitination. CNBr analysis of the receptors extracted from cells expressing the myc-tagged ubiquitin (data not shown) supports this view; attachment of the tagged ubiquitin to the 305–412 and 305–421 fragments retards the gel mobility, displacing it away from the 289–470 partial fragment. With this separation, it is clear that the level of mono-ubiquitinated 305–412 and 305–421 species present is significantly less than for the ubiquitinated forms of the CTD fragments, which retain Lys\textsuperscript{432}, indicating that mono-ubiquitination at lysyl sites mapping outside of the PEST-like sequence is minor. This conclusion is consistent with results for the 3K→R receptor, where the presence of a faint mono-ubiquitinated receptor band (Fig. 2) indicated that a low level of mono-ubiquitination was occurring to receptor sequences outside the PEST-like sequence.

In addition to the 305–470 partial product being present for all of the methionyl mutants, so too are the ubiquitinated forms of this partial fragment. These co-migrate with the identical species generated through complete digestion of the parental 2K→R receptor (Fig. 5C). The mono- and di-ubiquitinated forms of the 305–470 partial product are faintly apparent in the samples from Met\textsuperscript{412} and Met\textsuperscript{421} mutants, a reflection again of the increased amount of sample used for these two mutants (see above).

**Differential Effects of Energy Poisons on the Modification of Surface- and Vacuole-localized Receptor**—For Fig. 3, we noted that the wild-type and 3K→R receptors were modified to different extents. For the protease-shaving protocol (utilized for Fig. 3), the protease treatment of intact cells takes place in the presence of energy poisons, added to block the possibility of further membrane traffic. In Fig. 6, we have compared the modification status of wild-type Ste3p and Ste3(3K→R)p from extracts prepared from cells treated with or without energy poisons. When isolated from unpoisoned cells (lanes 2 of Fig. 6), wild-type Ste3p and Ste3(3K→R)p gave similar presentations, both migrated as a heterogeneous cluster of bands. For both also, phosphatase treatment collapses the heterogeneous clusters to a single, faster migrating species (lanes 3 of Fig. 6), indicating phosphorylation is the responsible modification. Treatment of cells with energy poisons prior to extract preparation results in some loss of the phosphoryl modification for both receptors. For the 3K→R receptor, this loss is more pronounced; it now co-migrates with phosphatase-treated receptor (Fig. 6, lane 1). In contrast, some portion of the wild-type receptor is able to resist the loss of phosphoryl modification during the energy poisoning incubations (Fig. 6, lane 1). The Δ413 receptor behaves like 3K→R; in the absence of poisons, the truncated receptor shows a heterogeneous phosphorylation, which is fully lost when receptor is isolated from poisoned cells (Fig. 6, lane 1). These different outcomes of the energy poisoning regimen appears to correlate with the different receptor localizations; the 3K→R and Δ413 receptors both localize to the plasma membrane (Fig. 3), while wild-type Ste3p localizes to the vacuole (18). Consistent with localization being the primary determinant, wild-type Ste3p trapped at the plasma membrane in end4 cells gives a similar energy poisoning result to the 3K→R receptor, i.e. complete loss of phosphoryl modification (data not shown). Energy poisons also affect receptor ubiquitination. Ste3p ubiquitination is easily observed for receptor accumulating either in the vacuole of the pep4Δ cells or at the cell surface in end4 cells (25). However, treatment of such cells with energy poisons results in a more complete loss of ubiquitin modification from the surface-localized receptor than from the vacuole-localized receptor; for the vacuole-localized receptor, some of the ubiquitinated receptor appears to be protected from the de-ubiquitinating effects of the energy poisons (data not shown).

One explanation for these phenomena relies on the differential energy requirements of the processes that add these modifications, versus the processes that remove them. Phosphorylation and ubiquitination both consume ATP, while removal of these modifications by endogenous phosphatases or ubiquitin proteases does not. Under energy poisoning conditions, the balance is expected to be shifted toward removal. When localized to the pep4Δ vacuole, the receptor may be less available to the cytoplasmic activity of phosphatases and ubiquitin proteases. Indeed, recent findings suggest that endocytosed substrates in yeast may be delivered wholly to the lumen of the vacuole, with the cytoplasmic domains of the endocytosed proteins being fully sequestered to the interior of the vacuole (28, 29).

**Ubiquitin Can Functionally Substitute for the Ste3p PEST-like Endocytosis Signal**—The analysis above demonstrated the presence of short multi-ubiquitin chains attached to Lys\textsuperscript{432} of Ste3(2K→R)p (Fig. 5C). However, previous experiments with Ste3p indicated that single ubiquitin moieties, rather than multi-ubiquitin chains, provide the key recognition elements that specify Ste2p uptake (2). To assess the role of mono- versus multi-ubiquitination in Ste3p endocytosis, we have followed the lead of Terrell et al. (2) and have constructed a fusion protein between Ste3p and ubiquitin. The Ste3-Ub fusion has the 7K→R ubiquitin (all lysines mutated to arginine) fused to the C terminus of a truncated Ste3p (Δ399), lacking its C-terminal 72 residues, including the PEST-like signal. The attached 7K→R ubiquitin lacks potential lysyl acceptor sites and thus is incapable of nucleating multi-ubiquitin chain formation. Furthermore, as the Ste3p constitutive endocytosis signal has been wholly deleted from the Ste3-Ub fusion, we can address questions regarding the sufficiency of the ubiquitin role that were not accessible by the original Ste2-Ub fusion studies (2). Does the fused ubiquitin functionally replace the PEST-like sequence for endocytosis? Restated, does the Ste3p PEST-like signal function solely for ubiquitination? Further, does the attached ubiquitin provide a sufficient signal to trigger uptake, or is ubiquitin recognized in conjunction with other peptidyl signals within the endocytic substrate?

Anti-Ste3p Western blots of extracts from cells expressing the Ste3-Ub fusion protein show the presence of a major new band migrating near the 50-kDa molecular mass expected for the Ste3-Ub fusion (Fig. 7A). Unexpectedly, however, in addition to the 50-kDa species, Ste3 antigen was also found to distribute heterogeneously throughout the gel lane, at molec-
B. indicated at as described for Fig. 6. The position of the major Ste3-Ub protein is 1) or following an additional incubation with energy poisons (from cells either immediately following the 2-h galactose induction by alanine (designated Ste3-Ub(G76A)). Protein extracts were prepared from cells either immediately prepared (panel A) or treated with energy poisons, and protein extracts were prepared and received an additional incubation in the presence of energy poisons as described for Fig. 6. The position of the major Ste3-Ub protein is indicated at left (arrow). 

Fig. 7. Expression and turnover of Ste3-Ub fusions. Strains having GAL1-driven STE3(Δ399)-Ub alleles replacing STE3 of isogenic wild-type (NDY343-derived), end4Δ-1+ (NDY344-derived), and pep4Δ (NDY372-derived) strains were subjected to the non-radioactive galactose-to-glucose pulse-chase regimen described for Fig. 2A, except that the cells were initially cultured at 25 °C and then shifted to 37 °C, 15 min prior to the completion of the galactose pulse. Following the 2-h period of galactose-induced Ste3-Ub expression, glucose was added and, at the indicated times, culture aliquots were collected. Protein extracts were either immediately prepared (−), or when indicated (+), cells received an additional incubation in the presence of energy poisons as described for Fig. 6. Protein extracts were subjected to SDS-PAGE and Western-blotted. 

| STE3 mutant | Half-lives* |
|-------------|-------------|
| Ste3p (wild-type) | 15 min |
| Ste3Δ399-486p | >2 h |
| Ste3(3K→R)p | >2 h |
| Ste3(Δ399)-Ub(wt) | 15 min |
| Ste3(Δ399)-Ub(7K→R) | 22 min |
| Ste3(wt)-Ub(7K→R) | 16 min |
| Ste3(3K→R)-Ub(7K→R) | 20 min |

* MATa strains isogenic to NDY343, except for having the above GAL1-driven STE3 mutants at the STE3 locus in place of ste3Δ. LEU2 were subjected to the non-radioactive pulse-chase described for Fig. 7C. Extracts were prepared from cells harvested at the time of glucose addition (0 min), and 30, 60, and 90 min afterward. The rate of Ste3 antigen loss was assessed via Western blotting with Ste3p-specific antibodies, followed by quantitative densitometry of the resulting films (as described under “Experimental Procedures”).

ular masses both below the 50-kDa band and extending well above to the gel origin (Fig. 7A, compare with ste3Δ lane). This unusual presentation, we believe, results not from modification of the Ste3-Ub fusion protein (for instance, by ubiquitination or phosphorylation), but rather from the ubiquitin-mimetic conjugation of C-terminal portions of this fusion protein to the various cellular substrates for ubiquitination. In a sense, the Ste3-Ub fusion resembles those ubiquitin constructs that have N-terminal epitope extensions. Such tagged ubiquitins efficiently substitute for wild-type ubiquitin in a variety of ubiquitin conjugation reactions (16, 22, 25, 30). The N-terminal extensions can be quite large; a glutathione S-transferase-Ub fusion with ubiquitin fused to the complete 26-kDa glutathione S-transferase enzyme may be efficiently conjugated to substrate (31). Thus, C-terminal fragments of the Ste3-Ub fusion (essentially, ubiquitin with N-terminally attached Ste3p portions) may be substituting for ubiquitin in the modification of other cellular substrates of ubiquitination. The heterogeneous gel smear then would reflect the huge variety of cellular proteins that normally serve as substrates for ubiquitination.

The covalent linkage of ubiquitin to substrate joins the C-terminal Gly76 of ubiquitin to the e-amino group of a substrate lysyl side chain. Unlike alterations at the ubiquitin N terminus, which generally do not affect conjugation of ubiquitin to substrate (see above discussion), alteration of the Gly75 and Gly76 C-terminal residues might be expected to be more disabling. We have constructed two mutations at the Ste3-Ub C terminus: Ste3-Ub(Δ175, 76) having the two C-terminal ubiquitin glycine codons removed and Ste3-Ub(G76A) for which Gly76 of ubiquitin is changed to alanine. Both mutations were found to both profoundly reduce the heterogeneous gel mobility associated with the Ste3-Ub fusion and concomitantly, increase the proportion of the Ste3 antigen migrating at the 50-kDa position (Fig. 7A). The effectiveness of these two C-terminal ubiquitin mutations in blocking the heterogeneous re-distribution of Ste3 antigen through the gel lane supports the explanation that this heterogeneity is a consequence of the ubiquitin-mimetic conjugation of Ste3-Ub fragments to cellular ubiquitination substrates.

We have also tested the energy poisoning regimen used for Fig. 6 for effects on the heterogeneous gel distribution of the Ste3-Ub fusion protein (Fig. 7A). This treatment, which can result in the loss of Ste3p modifications, might also reverse the attachment of Ste3-Ub to other cellular protein. Indeed, we find that this is the case: energy poisons fully reverse the heterogeneous distribution of Ste3 antigen throughout the gel lane (Fig. 7A).

As a test of the functionality of these fusions in endocytosis, we have first examined effects on constitutive turnover. The truncated Δ399 receptor (equivalent to the Ste3 portion of the Ste3-Ub fusion) is not subject to constitutive turnover (Table II) and stably accumulates at the cell surface (1). In contrast, this
truncation having the 7K→R ubiquitin fused to its C terminus clearly does turn over (Fig. 7B). Thus, the added ubiquitin restores turnover to the signal-deleted receptor. Furthermore, rapid turnover kinetics are unaffected when the C-terminal G76A mutation is introduced (Fig. 7B). Likewise, the addition of the energy poisoning regimen to the experimental protocol also does not affect the outcome; rapid turnover is still seen (Fig. 7B). Given the lack of effect of the energy poisoning treatment on the experimental result, this regimen has been routinely incorporated into the following experiments that test different Ste3-Ub fusions.

Rapid constitutive turnover of the wild-type α-factor receptor depends on the endocytic delivery of surface receptor to the vacuole for degradation. Ste3p turnover is blocked in mutant cell backgrounds that are defective for either endocytosis (e.g. end4 cells) or for vacuolar protease activity (e.g. pep4A cells) (Fig. 7C). We find that Ste3-Ub turnover shows the same requirements, being blocked in both end4 and pep4A cells (Fig. 7C). In addition, we have used the protease-shaving protocol to examine the localization of the Ste3-Ub fusion in these two cell backgrounds (Fig. 8). In end4 cells, Ste3-Ub accumulates at a locale where it is largely sensitive to digestion by the added proteases, probably the plasma membrane. In pep4A cells, Ste3-Ub accumulates within some intracellular compartment (probably the vacuole) where it resists digestion by the extracellular proteases (Fig. 8).

We conclude that, as with wild-type Ste3p, turnover of the Ste3-Ub fusion protein also depends on its endocytic transport from the cell surface to the vacuole. A further conclusion is that the Ste3p constitutive endocytosis signal may be fully functionally replaced by the added mono-ubiquitin. With ubiquitin pre-attached to the receptor, the need for the endocytosis signal in endocytosis is bypassed; it has become dispensable, suggesting that this signal functions solely in endocytosis for directing ubiquitination. Furthermore, we can conclude that ubiquitin functions alone to trigger uptake. No other receptor sequence or signal is required in conjunction.

Our results also confirm those of Terrell et al. (2), which indicated that multi-ubiquitin chain formation is not required for endocytosis; rather, mono-ubiquitin provides the key elements recognized by the endocytic apparatus in initiating uptake. While mono-ubiquitination of Ste3p suffices, it has been suggested from studies of the uracil permease Fur4p and of the general amino acid permease Gap1p that short multi-ubiquitin chains, having Lys63ubiquitin-ubiquitin linkages may play a role in stimulating the rate of uptake (31, 32). Indeed, our analysis of Ste3p ubiquitination showed the presence of short multi-ubiquitin chains associated with some of the receptors (Fig. 5C). Furthermore, while turnover of the Ste3-Ub fusion is rapid (t_1/2 of 22 min; Table II), it is somewhat less rapid than the turnover of wild-type Ste3p (t_1/2 = 15 min; Table II). Perhaps this rate difference reflects the potential for multiple ubiquitin attach-
DISCUSSION

The Lysyl Acceptor Sites for Ubiquitin Addition—The three lysines that mapped within the PEST-like endocytosis/ubiquitination signal provided an obvious starting point in our efforts to identify the ubiquitin acceptor sites associated with Ste3p rapid constitutive endocytosis. All combinations of Lys → Arg replacements were constructed and tested for effects both on receptor turnover and ubiquitination. Results support a model in which the three lysines function redundantly as sites for ubiquitin conjugation. Receptor mutants having one or two of the three lysines replaced showed, at most, partial defects for ubiquitination and turnover. Complete impairment was seen only for receptors in which all three lysines had been removed. This redundancy implies a certain plasticity in terms of which of the three is used as the acceptor site. This plasticity is limited, however, as it clearly does not extend to the 21 lysine residues that map outside of the PEST-like sequence to other cytoplasmic portions of the receptor protein; these other lysines may not substitute for the loss of the three endocytosis signal lysines in the 3K→R mutant receptor, indicating that they may not be able to serve as ubiquitin acceptors.

Of the three PEST-like sequence lysines, Lys\textsuperscript{432} normally play the most prominent role; K432R mutant receptors are partially impaired for both ubiquitination and turnover, and K424R,K453R receptors, which retain Lys\textsuperscript{432}, show wild-type ubiquitination and turnover. It is likely that Lys\textsuperscript{432} normally serves as the primary ubiquitination site with Lys\textsuperscript{424} and Lys\textsuperscript{453} serving as alternative, secondary attachment sites.

Results from CNBr fragmentation of ubiquitinated Ste3p largely confirm results from Lys → Arg mutagenesis, indicating that the PEST-like sequence does indeed provide the site for the much of the ubiquitination that is associated with constitutive endocytosis. In addition, this analysis provides the first direct biochemical evidence for multi-ubiquitin chains associated with endocytosis. Our analysis of Ste3p ubiquitination was simplified by concentrating on the K424R,K453R mutant receptor, which retained just the Lys\textsuperscript{432} acceptor site. CNBr fragments that retained Lys\textsuperscript{432}, \textit{i.e.} the 305–441, 305–452, and 305–470 CTD fragments, were found to be modified with 0, 1, 2, or 3 ubiquitin moieties (Fig. 5C). Fragments lacking Lys\textsuperscript{432}, \textit{i.e.} 305–412 and 305–421, showed a commensurate loss of the di- and tri-ubiquitinated species. From this we conclude that Lys\textsuperscript{432} is modified with a di-ubiquitin chain and a subset may also be modified with a tri-ubiquitin chain. The uncertainty regarding the tri-ubiquitin chain relates to the low level mono-ubiquitination of non-PEST-like sequence lysines, apparent for both the 3K→R receptor (Fig. 2, C and D, and Fig. 4B) and for the K424R,K453R receptors (Fig. 5C). The tri-ubiquitinated CNBr fragments could consist of two moieties linked in series at Lys\textsuperscript{432} with the third ubiquitin attached to a separate lysyl residue, elsewhere in the receptor CTD.

Functional Replacement of the Endocytosis Signal by Ubiquitins—Our finding of a short multi-ubiquitin chain attached to Lys\textsuperscript{432}, we were interested to test if such multi-ubiquitin chains are required for Ste3p endocytosis. Previous experiments for Ste2p had indicated that endocytosis differs from proteosomal targeting in that single ubiquitin moieties, and not multi-ubiquitin chains, provide the targeting information for internalization (2). Repeating these experiments with Ste3p, with the construction of translational fusions between Ste3p and a 7K→R ubiquitin, confirms the Ste2p findings (2). Like Ste2p, multi-ubiquitination is not required for endocytosis. Mono-ubiquitin provides the necessary targeting information.

In addition to confirming conclusions made for Ste2p (2), our results with the Ste3p fusions also extend these conclusions. The Ste3Δ399)-Ub fusions lack all Ste3p signals and sequences required for constitutive endocytosis (1). The ability of the attached ubiquitin to functionally replace these sequences indicates the sufficiency of the mono-ubiquitin in specifying
uptake. This leads us to the important conclusion that ubiquitin functions alone to trigger uptake, not in conjunction with additional receptor sequences or signals. Attachment of a single ubiquitin moiety to a yeast plasma membrane protein is all that is required to specify uptake. A further, more definitive test of this conclusion might involve construction of 7K→R ubiquitin translational fusions with a plasma membrane protein such as Pma1p which normally does not undergo endocytosis. If mono-ubiquitin indeed functions alone to trigger uptake, then such a Pma1-Ub(7K→R) fusion should undergo endocytosis.

**Ubiquitination Signals: Proteosomal Targeting Versus Endocytosis**—Our finding that ubiquitin acceptor sites map within the confines of the PEST-like signal perhaps seems unremarkable. For phosphorylation signals, the phosphate acceptor site (i.e. Ser, Thr, or Tyr) generally is a landmark feature within the consensus used by the kinase for recognition. This, however, generally is not true for ubiquitination signals that have been characterized to date, these being the signals associated with proteosomal targeting. The idealized ubiquitination signal has been described as being bipartite, with the recognition element and the ubiquitin attachment site, being separate and separable (35). The two elements may be distant from each other in the primary sequence and extreme cases, may even localize to distinct polypeptide subunits (36). Although formulated within the context of N-end Rule substrates (35), this bipartite model also appears to apply to a variety of ubiquitination signals; the recognition element, be it a PEST sequence or a destruction box, generally does not include the lysyl attachment site (37). Indeed, for a number of proteosomal substrates, investigation of the lysyl acceptor site use has uncovered a shocking level of plasticity; in several cases, any lysine within the substrate protein is capable of functioning as the ubiquitin acceptor site (38–40).

The separation of recognition element and acceptor site seen for proteosomal substrates may relate to the need for multi-ubiquitin chain assembly. Attachment of an initial ubiquitin to a site embedded within the recognition element could have the consequence of disrupting the recognition element, potentially inhibiting subsequent ubiquitin addition. The addition of the multiple ubiquitin moieties, as required for the assembly of a multi-ubiquitin chain, may require a prolonged recognition of the signal by the E2 and/or E3 enzymes catalyzing this addition. For endocytosis, the multi-ubiquitin chain is not required (this work; Ref. 2). Furthermore, it has been suggested that recognition of substrate mono- versus multi-ubiquitination may be the key for deciding which of the two outcomes is invoked: endocytosis or proteosomal degradation (2). The strategy employed by the α-factor receptor wherein the acceptor sites are embedded within the recognition element may help to assure the endocytic outcome. If attachment of the first ubiquitin moiety disrupts the PEST-like recognition element and impairs its use for subsequent rounds of recognition, then ubiquitin addition would be self-limiting, with only one or several ubiquitin moieties being added.

**Attachment of Multiple Ubiquitins Increases Uptake Rate**—In addition to being subject to mono-ubiquitination, analysis of Ste3p ubiquitination shows that a subset of the receptor population is subject to bi-ubiquitination and perhaps to tri-ubiquitination as well. While the Ste3-Ub fusion results indicate that mono-ubiquitin suffices for initiating uptake, these same results also suggest a possible role for multi-ubiquitination in augmenting the rate of uptake. First, the Ste3Δ(399)-Ub(wt) fusion showed slightly faster turnover than did the Ste3Δ(399)-Ub(7K→R) fusion (Fig. 9 and Table II). As the Ste3Δ(399)-Ub(wt) fusion retains the ubiquitin lysines, its increased turnover may reflect secondary ubiquitination of the translationally attached ubiquitin moiety. A more striking example of enhanced endocytosis due to multi-ubiquitination is evident with the Ste3Δ(399)-Ub(7K→R) fusion; this fusion turns over with a t1/2 of 9 min (Table II), significantly faster than wild-type Ste3p (t1/2 = 15 min; Table II). For this fusion, there apparently is a synergy between the natural ubiquitination of the receptor (specified by the PEST-like signal) and the artificial ubiquitination (the translationally fused 7K→R ubiquitin). The added ubiquitin moiety fused to the receptor C terminus stimulates uptake beyond the rate seen for the wild-type receptor.

Based upon the effects of K63R ubiquitin mutants on Gap1p and Fur4p endocytosis, it has been suggested that short multi-ubiquitin chains with Lys63 ubiquitin-ubiquitin linkages may serve to enhance the rate of uptake (32, 33). For Ste3p, we now supply additional evidence for a stimulatory effect of multi-ubiquitination. However, given the functionality of the receptor fusions, which are obligately mono-ubiquitinated (this work; Ref. 2), we feel that the key recognized feature for endocytosis must be the mono-ubiquitin itself, not a linked chain. From this perspective, we would expect that multiple individual ubiquitins attached to separate substrate lysyl residues would prove just as stimulatory for endocytosis as linking these ubiquitins together in the form of a multi-ubiquitin chain.

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Ubiquitination of the PEST-like Endocytosis Signal of the Yeast a-Factor Receptor
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